Universitat de Lleida

Estudio de las bases fisiológicas, bioquímicas y moleculares de la maduración en peras y su relación con la aparición del escaldado superficial

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http://hdl.handle.net/10803/670215

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TESIS DOCTORAL

Estudio de las bases fisiológicas, bioquímicas y moleculares de la maduración en peras y su relación con la aparición del escaldado superficial

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Memoria presentada para optar al grado de Doctora por la Universitat de Lleida

Programa de **Doctorado en Ciencia y Tecnología Agraria y Alimentaria**

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Lleida, octubre de 2020

Los estudios presentados en esta tesis doctoral se han desarrollado en los laboratorios de Fisiología y Tecnología del programa de Poscosecha del Fruitcentre-IRTA (Institut de Recerca i Tecnologia Agroalimentàries) de Lleida.

Para la realización de este trabajo se recibió financiación del Ministerio de Economía, Industria y Competitividad (MINECO) a través del proyecto AGL2017-87923-R; de la Agència de Gestió d'Ajuts Universitaris i de Recerca (AGAUR), conjuntamente con el Fondo Social Europeo ("El FSE invierte en tu futuro") para la contratación de personal investigador novel (2017FI_B_00639) y también del programa CERCA (Centres de Recerca de Cantalunya) de la Generalitat de Catalunya.

A Servanda y Ángel, porque todo lo que soy es gracias a vosotros

"El momento que da más miedo es siempre justo antes de empezar"

-Stephen King-

"Debes aprender a estar tranquilo en medio de la actividad y estar intensamente vivo durante el reposo"

-Indira Gandhi-

"Siempre parece imposible hasta que se hace"

-Nelson Mandela-

Quien me conoce bien sabe que soy una sentimental y que, por lo tanto, este apartado no podía faltar en mi tesis. Además, el hecho de escribirla durante el confinamiento de una pandemia mundial (¡Quién me lo iba a decir!) ha hecho que reflexionase y valorase más algunas cosas que quizás antes no valoraba tanto.

En primer lugar, me gustaría agradecer al IRTA-Fruitcentre en general y en especial al Dr. Christian Larrigaudière por haberme dejado formar parte del grupo durante estos más de tres años en los cuales he aprendido muchísimo tanto a nivel personal como profesional, gracias por tu cercanía y por tu apoyo. También al Dr. Jordi Giné por estar siempre al pie del cañón, gracias por contagiarme toda esa energía y hacer de líder y compañero a partes iguales. Gracias también a la Dra. Gemma Echeverría por ejercer de madre postiza, me quedo con tu positividad y la alegría que desprendes. A la Dra. Luisa López, gracias por acceder a ser la tutora académica de este trabajo y estar disponible siempre que te he necesitado.

Gracias a Eli y Dolors por haber sido mis manos y mis pies durante todo este tiempo; gracias por vuestra paciencia, por todo lo que he aprendido de vosotras y por todos los buenos momentos que hemos pasado juntas… ¡Valéis mucho! Además de ellas, gracias a toda la gente que me ha echado una mano en cada campaña y que ha estado conmigo codo con codo en el laboratorio, ya que todos los ensayos de esta tesis no los podría haber realizado sin ellos; gracias a Albert, Raquel, Cris, Joana y Gemma López, ha sido un verdadero placer haber podido trabajar con vosotr@s. También gracias a toda la gente que ha pasado por el laboratorio y que de una forma u otra me ha aportado su granito de arena.

No me puedo olvidar de mis compañeros de sala… ¡Qué hubiera sido de mí sin esa salita! Ha sido nuestro despacho, pero también ha hecho de confesionario, gimnasio, y centro de yoga y meditación para los momentos de mayor estrés. Creo que de esta sala he conocido a gente que, a día de hoy, puedo considerar amigas. Gracias a Lorena por todos los momentos que hemos pasado juntas en Lleida, me alegra que sigamos tan en contacto a pesar del tiempo que ha pasado y lo poco que nos vemos… ¡Te debo una visita! Mientras tanto, siempre nos quedarán nuestros *podcasts* semanales. Gracias a Núria Baró y Anna por cada plan improvisado durante estos tres años y pico, me ha encantado compartir todos esos momentos con vosotras y espero que todavía nos queden muchos más planes juntas. Marta, Dani, Jordi, Ioli, Pablo y Lucía, habéis sido unos compañeros inmejorables, gracias a vosotros una entraba en la sala con una

sonrisa de oreja a oreja. Gracias también a Núria Vall-llaura, Xesca y Sandra por los buenos momentos vividos tanto dentro como fuera de IRTA.

A tots vosaltres... No us imagineu quant us trobaré a faltar!

Dejando Lleida a un lado quiero agradecer a mis amigas de Cáceres su paciencia y el entender que no las podía ver tanto como me hubiese gustado, gracias por haber sacado un huequito para vernos cada vez que he ido a Cáceres. Tampoco podía faltar mi "familia" de Salamanca, con vosotros empezó todo hace ya más de 10 años y ojalá no termine nunca; gracias por cada reencuentro que ha sido indispensable para coger fuerzas durante este tiempo.

Yendo a la parte más íntima, gracias a la Paquiña por desearme lo mejor día a día y por inculcarme que sólo se es joven una vez, que la vida pasa volando y hay que disfrutar cada momento al máximo. Gracias también a mi Tata Quini, de ti he aprendido que, aunque las cosas se vuelvan oscuras, la sonrisa es siempre lo último que se pierde y tú más que nadie me lo has demostrado.

A mis padres, ¡cómo no!, por haber estado ahí siempre y por todo el apoyo que me habéis dado en cada momento de mi vida, sabéis que todo esto no hubiera sido posible sin vosotros. ¡Gracias por el gran esfuerzo que habéis hecho en comprender todo lo que supone hacer una tesis! Esta va por vosotros…

Por último, esta tesis también se la quiero dedicar a Ángel por ser un pilar fundamental en mi vida y haber sido un apoyo incondicional durante estos más de 3 años en Lleida y, sobre todo, en esta última etapa. Gracias por demostrarme que, si se quiere, se puede, y que la distancia no es más que un número.

A TODOS… ¡GRACIAS DE CORAZÓN!

ÍNDICE

RESUMEN/RESUM/SUMMARY

El escaldado superficial es una de las principales alteraciones fisiológicas en poscosecha de manzana y pera. A pesar de que los síntomas aparecen, en general, tras un largo periodo de almacenamiento en frío, la susceptibilidad de los frutos a esta alteración se determina en campo, se induce en los primeros meses de conservación en frío y depende de la variedad. Sin embargo, las bases bioquímicas y moleculares que determinan dicha susceptibilidad o cómo la conservación en frío regula la inducción de la alteración son en gran medida desconocidas, y en especial en el caso de la pera. Por esto, el principal objetivo de esta tesis fue estudiar las bases fisiológicas, bioquímicas y moleculares de la maduración en peras y su relación con el desarrollo del escaldado superficial. En el Capítulo 1 se estudiaron los cambios hormonales y sus interacciones (*cross-talk*) durante el desarrollo en dos variedades de pera para ver cómo podían afectar al posterior comportamiento poscosecha del fruto. Los resultados mostraron que el etileno no es la única hormona involucrada en el desarrollo y la maduración de la pera, sino que existe un complejo *cross-talk* con otras hormonas, entre las que destacan el ácido abscísico (ABA), el ácido 3-indolacético (IAA) y la giberelina 1 (GA_1). En los Capítulos 2 y 3 se estudiaron los patrones de maduración en árbol y tras la cosecha de tres variedades con comportamientos poscosecha muy distintos. Se comprobó que, de las tres variedades, "Blanquilla" era la única capaz de madurar en el árbol y que esta capacidad se asocia a una acumulación de sacarosa, la cual parece inducir la producción de etileno. La inducción de la maduración en esta variedad después de cosecha, se debe en primer lugar a un proceso oxidativo que posteriormente parece inducir una serie de respuestas etileno-dependientes. Por otro lado, la incapacidad de madurar en el árbol de las variedades "Conference" y "Flor d'Hivern" refuerza la hipótesis del *tree factor*, la cual se refiere a la existencia de una molécula suministrada por la planta capaz de inhibir el proceso de maduración mientras el fruto está unido a ella. Esta molécula, aún desconocida, podría ser la responsable de aumentar la resistencia del fruto a sufrir un daño oxidativo, impidiendo así su maduración. Pasando a la fase poscosecha, se buscó un marcador en cosecha capaz de predecir la susceptibilidad al escaldado superficial asociada a distintos estados de madurez (Capítulo 4). Nuestros resultados mostraron que el ACC, la actividad ACS y la expresión del gen *PcAFS1* son buenos marcadores correlacionados positivamente con la incidencia de escaldado superficial en "Blanquilla"; sin embargo, la falta de correlación observada en "Flor d'Hivern" con los parámetros estudiados se debió principalmente al metabolismo inusual del etileno, sugiriendo en este caso que la causa del desorden se debe a un proceso oxidativo etileno-independiente. Por último, con el objetivo de estudiar la regulación a nivel bioquímico y molecular del etileno y el α-farneseno durante las primeras semanas de almacenamiento en frío, en el Capítulo 5 se trabajó con diferentes inhibidores específicos de estas vías metabólicas: el 1-metilciclopropeno (1-MCP) y la lovastatina. Con estos tratamientos se ha podido mostrar que la alteración en "Blanquilla" está íntimamente relacionada con el etileno y el α-farneseno y se controla tanto con el 1-MCP como con la lovastatina. Por otro lado, el desorden en "Flor d'Hivern" se controla solamente con el 1-MCP, el cual parece actuar potenciando la capacidad de aclimatación del fruto al frío. Por último, la ineficacia del 1-MCP y de la lovastatina para el control de la alteración en "Conference", sugiere la presencia de una alteración de etiología diferente al escaldado.

L'escaldat superficial és una de les principals alteracions fisiològiques en postcollita de poma i pera. Tot i que els símptomes apareixen, en general, després d'un llarg període d'emmagatzematge en fred, la susceptibilitat dels fruits a aquesta alteració es determina en camp, s'indueix en els primers mesos de conservació en fred i depèn de la varietat. No obstant això, les bases bioquímiques i moleculars que determinen aquesta susceptibilitat o la manera com la conservació en fred regula la inducció de l'alteració són en gran mesura desconegudes, i especialment en el cas de la pera. Per això, el principal objectiu d'aquesta tesi va ser estudiar les bases fisiològiques, bioquímiques i moleculars de la maduració en peres i la seva relació amb el desenvolupament de l'escaldat superficial. En el Capítol 1 es van estudiar els canvis hormonals i les seves interaccions (*cross-talk*) durant el desenvolupament de dues varietats de pera per veure com aquestes podien afectar el posterior comportament postcollita del fruit. Els resultats van mostrar que l'etilè no és l'única hormona involucrada en el desenvolupament i la maduració de la pera, sinó que hi ha un complex *cross-talk* amb altres hormones, entre les quals destaquen l'àcid abscísic (ABA), l'àcid 3-indolacétic (IAA) i la giberel·lina 1 (GA_1). En els Capítols 2 i 3 es van estudiar els patrons de maduració en arbre i en postcollita de tres varietats amb comportaments postcollita molt diferents. Es va comprovar que, de les tres varietats, "Blanquilla" era l'única capaç de madurar a l'arbre i que aquesta capacitat s'associava a un increment de sacarosa que sembla induir la producció d'etilè. Per altra banda, la inducció de la maduració en aquesta varietat després de la collita, es deu en primer lloc a un procés oxidatiu que posteriorment sembla induir una sèrie de respostes etilè-dependents. D'altra banda, la incapacitat de madurar a l'arbre de les varietats "Conference" i "Flor d'Hivern" reforça la hipòtesi del *tree factor*, l'existència d'una molècula subministrada per la planta capaç d'inhibir el procés de maduració mentre el fruit està unit a ella. Aquesta molècula, encara desconeguda, podria ser la responsable d'augmentar la resistència del fruit a patir un dany oxidatiu, impedint així la seva maduració. Passant a la fase postcollita, es va buscar un marcador en collita capaç de predir la susceptibilitat a l'escaldat superficial associada a diferents estats de maduresa (Capítol 4). Els nostres resultats van mostrar que l'ACC, l'activitat ACS i l'expressió del gen *PcAFS1* són bons marcadors correlacionats positivament amb la incidència d'escaldat superficial en "Blanquilla"; però la falta de correlació observada en "Flor d'Hivern" amb els paràmetres estudiats es va deure principalment a el metabolisme inusual de l'etilè, suggerint en aquest cas que la causa del desordre es deu a un procés oxidatiu etilè-independent. Finalment, amb l'objectiu d'estudiar la regulació a nivell bioquímic i molecular de l'etilè i el α-farnesè durant les primeres setmanes d'emmagatzematge en fred, al Capítol 5 es va treballar amb diferents inhibidors específics d'aquestes vies metabòliques: el 1-metilciclopropè (1-MCP) i la lovastatina. Amb aquests tractaments s'ha pogut mostrar que l'alteració en "Blanquilla" està íntimament relacionada amb l'etilè i el α-farnesè i es controla tant amb l'1-MCP com amb la lovastatina. D'altra banda, el desordre observat en "Flor d'Hivern" sembla estar relacionat amb un dany per fred i l'1-MCP sembla controlar el desordre potenciant la capacitat d'aclimatació del fruit al fred. Finalment, la ineficàcia de l'1-MCP i de la lovastatina per al control de les alteracions en "Conference", suggereix la presència d'una alteració d'etiologia diferent a l'escaldat.

Superficial scald is one of the main postharvest physiological disorders in apples and pears. Despite symptoms appear after long-term cold storage, the susceptibility of the fruit is determined pre-harvest, is induced during the first months of cold storage and it strongly depends on the cultivar. However, the biochemical and molecular basis determining the fruit susceptibility to superficial scald or the cold-induced regulation of the disorder are largely unknown, especially in pear fruit. Hence, the main objective of this thesis was to study the physiological, biochemical and molecular basis of pear maturation and ripening and its relationship with superficial scald development. In Chapter 1, the hormonal changes and their interactions ('cross-talk') during fruit development were studied in two pear cultivars to clarify how they affect to the postharvest behaviour. The results showed that ethylene is not the only hormone involved in pear development and ripening, but rather its complex interaction or cross-talk with other hormones, and specifically abscisic acid (ABA), indole-3-acetic acid (IAA) and gibberellin 1 (GA_1). In Chapters 2 and 3, on- and off-tree ripening patterns were studied in three cultivars with different postharvest behaviours. From the studied varieties, 'Blanquilla' was the only cultivar able to ripen on-tree and this ability was associated to an enhanced sucrose accumulation, which may participate in the induction of the fruit ethylene production. Off-tree ripening in this cultivar was firstly associated to an oxidative-mediated process, which then seemed to trigger the ethylene-dependent responses. On the other hand, the inability of 'Conference' and 'Flor d'Hivern' pears to ripen on the tree reinforces the hypothesis of the existence of a molecule supplied by the plant and capable to inhibit the ripening process ('tree factor'). This molecule, yet unknown, may be responsible of increasing the fruit resistance to an oxidative damage, thereby inhibiting ripening. Then, a biochemical or molecular marker able to predict the scald susceptibility of the fruit at harvest was searched at different stages of maturity (Chapter 4). Our results showed that ACC, ACS activity and *PcAFS1* gene expression were good markers positively correlated with superficial scald susceptibility in 'Blanquilla' pear. The lack of correlation of the studied parameters in 'Flor d'Hivern' was due to the observed unusual ethylene metabolism, suggesting an ethylene-independent oxidative process as the main cause of the storage disorder found in this cultivar. Finally, with the objective to study the biochemical and molecular regulation of ethylene and α-farnesene during the first weeks of cold storage, in Chapter 5, fruit were treated with specific inhibitors of these metabolic pathways: 1-methylcyclopropene (1- MCP) and lovastatin. The storage disorder observed in 'Blanquilla' pear was strongly associated to ethylene and α-farnesene metabolism, and it can be controlled by either 1-MCP or lovastatin. However, the physiological disorder observed in 'Flor d'Hivern' seems to be related to a typical chilling injury disorder and 1-MCP may control its development by enhancing the cold-acclimation capacity of the fruit. Finally, the inefficacy of 1-MCP and lovastatin in the controlling scald-like disorders in 'Conference' pear suggests a disorder with a complete different etiology than superficial scald.

ABREVIATURAS

1-MCP	1-metilciclopropeno
AAT	Alcohol aciltransferasa
ABA	*Abscisic acid* Ácido abscísico
ACC	*1-aminocyclopropane-1-carboxylic acid* Ácido 1-aminociclopropano-1-carboxílico
ACO	ACC oxidasa
ACS	ACC sintasa
ADH	Alcohol deshidrogenasa
AFS	α-farneseno sintasa
ANOVA	Análisis de varianza
APX	Ascorbato peroxidasa
AsA	Ascorbato
AVG	Aminoetoxivinilglicina
BR	Brasinoesteroide
CAT	Catalasa
CH	*Commercial harvest* Cosecha comercial
CHD	*Commercial harvest date* Fecha de cosecha comercial
CK	Citoquinina
CT	*Conjugated triene* Trieno conjugado
CTH	*Conjugated triene hydroperoxide* Hidroperóxido de trieno conjugado
CTol	*Conjugated trienol* Trienol conjugado
CTIFL	*Centre Technique Interprofessionnel des Fruits et Légumes* Centro técnico interprofesional de frutas y hortalizas

DACH	*Days after commercial harvest* Días después de cosecha comercial
DAFB	*Days after full bloom* Días después de plena floración
DARP	Departament d'Agricultura, Ramaderia, Pesca i Alimentació
DCA	*Dynamic controlled atmosphere* Atmósfera dinámica controlada
DDPF	Días después de plena floración
DHA	Dehidroascorbato
DHAR	Dehidroascorbato reducatasa
DOC	Día óptimo de cosecha
DPA	Difenilamina
DTT	Ditiotreitol
EDTA	*Ethylenediaminetetraacetic acid* Ácido etilendiaminotetraacético
EIN	*Ethylene insensitive (protein)* (Proteína) insensible al etileno
ERF	*Ethylene Response Factor* Factor de respuesta a etileno
ESI	*Electrospray ionization* Ionización por electrospray
ETR	*Ethylene receptor* Receptor de etileno
FAOSTAT	*Food and Agriculture Organization of the United Nations (Statistical database)* Organización de las Naciones Unidas para la alimentación y la agricultura (Base de datos estadísticos)
FDS	Farnesil difosfato sintasa
FID	*Flame ionization detector* Detector de llama iónica
FPP	Farnesil difosfato
FRAP	*Ferric reducing antioxidant power* Poder antioxidante reductor del hierro

GA	Giberelina
GACC	γ-glutamil-ACC
GC	*Gas cromatography* Cromatografía de gases
GSH	Glutatión reducido
GST	Glutatión-S-transferasa
GPP	Geranil difosfato
GR	Glutatión reductasa
HMGR	Hidroximetilgrlutaril-coA reductasa
HPLC	*High performance liquid cromatography* Cromatografía líquida de alta resolución
IAA	*Indole-3-acetic acid* Ácido 3-indolacético
ILOS	*Initial low oxygen stress* Estrés inicial con bajo oxígeno
IPP	Isopentenil difosfato
JA	*Jasmonic acid* Ácido jasmónico
JA-ACC	Jasmonil-ACC
JAR1	*Jasmonate resistant 1* (Comúnmente conocida como la enzima jasmonato sintetasa)
LOX	Lipoxigenasa
LSD	*Least signifcance difference* Mínima diferencia significativa
MACC	Malonil-ACC
MAPA	Ministerio de Agricultura, Pesca y Alimentación
MDA	Malondialdehído
MDHAR	Monodehidroascorbato reductasa
MEP	*Methylerythritol phosphate pathway* Ruta del metileritritol fosfato
MNE	*Mean normalised expression* Expresión media normalizada

MOPS	*3-(N-morpholino)propanesulfonic acid* Ácido 3-(N-morfolino)propanosulfónico
MS	*Mass spectrometry* Espectrometría de masas
MTA	5-metiltioadenosina
PCA	*Principal component analysis* Análisis de componentes principales
PG	Poligalacturonasas
PME	Pectinmetilesterasa
POX	Peroxidasa
PPO	Polifenoloxidasa
PVP	Polivinilpirrolidona
PVPP	Polivinilpolipirrolidona
RH	*Relative humidity* Humedad relativa
RNA	*Ribonucleic acid* Ácido ribonucleico
RT-qPCR	*Real time – quantitative polymerase chain reaction* Reacción en cadena de la polimerasa cuantitativa en tiempo real
S6PDH	Sorbitol 6-fosfato deshidrogenasa
SA	*Salicylic acid* Ácido salicílico
SAM	S-adenosil-L-metionina
SAR	*Systemic acquired resistance* Respuesta sistémica adquirida
SDH	Sorbitol deshidrogenasa
SI	*Starch index* Índice de almidón
SOD	Superóxido dismutasa
SPME	*Solid phase micro extraction* Micro-extracción en fase sólida

TA	*Titratable acidity* Acidez titulable
TBA	*Thiobarbituric acid* Ácido tiobarbitúrico
TBARS	*Thiobarbituric acid reactive substrates* Sustancias reactivas del ácido tiobarbitúrico
TCA	*Trichloracetic acid* Ácido tricloroacético
TSS	*Total soluble solids* Contenido en sólidos solubles
UHPLC	*Ultra HPLC* Ultra HPLC
UA	*Unit of activity* Unidad de actividad
ULO	*Ultra low oxygen* Oxígeno ultra bajo
xULO	*Extreme ULO* ULO extremo

INTRODUCCIÓN

- Conceptos generales -

1. Fruta de pepita

La fruta de pepita (uva, pera y manzana) representa un 18,1 % del total de la fruta consumida en España, después de los cítricos (28,1 %) y las frutas exóticas (19,5 %) (MAPA, 2019a). China es el mayor productor de pera del mundo con casi un 70 % de la producción, mientras que España se encuentra en el décimo lugar (Fig. 1) (FAOSTAT, 2018). En el año 2018 se produjeron 332.319 toneladas de pera en España, casi el 40 % en Cataluña (MAPA, 2019b). De las más de 129.000 toneladas producidas en Cataluña en el año 2018, casi 124.000 se produjeron en Lleida, de ahí que sea la provincia con mayor producción de peras de Cataluña y España (DARP, 2019a).

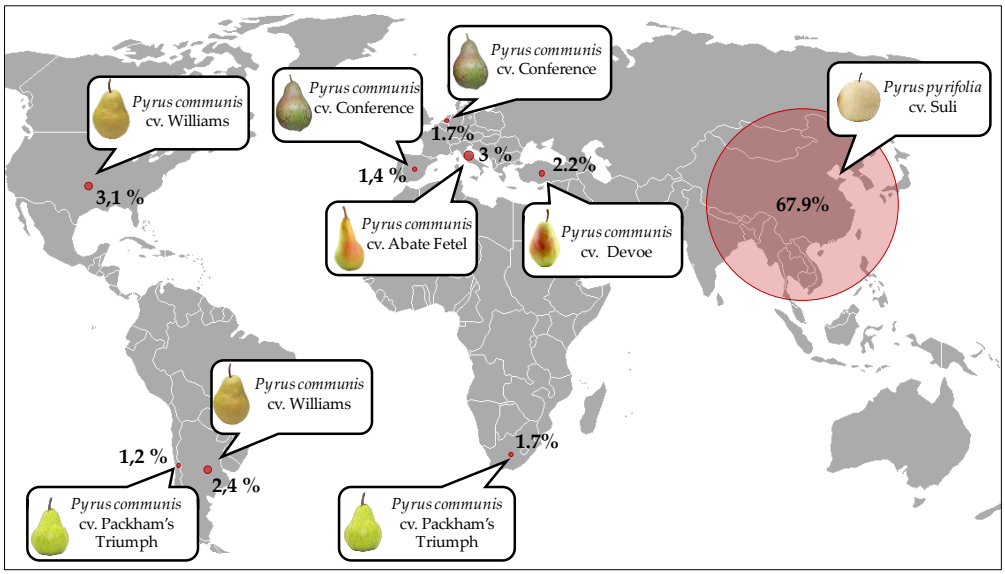

Figura 1. Principales países productores de pera y las variedades más producidas en ellos (Datos FAOSTAT, 2018).

1.1. Variedades de pera

El peral pertenece al género *Pyrus*, dentro de la familia de las Rosáceas, y existen unas 30 especies dentro de este género, las cuales se dividen de manera general en peras occidentales y peras asiáticas (Fig. 2). Dentro de las primeras cabe destacar la especie *Pyrus communis* (mayoritaria en Europa por lo que comúnmente se alude a ella como pera europea) y dentro de las peras asiáticas nos encontramos con: *Pyrus pyrifolia* (también conocida como pera Nashi o japonesa) y *Pyrus ussuriensis* (o pera china). A pesar de denominarse peral europeo, la especie *Pyrus communis* se encuentra

distribuido por todo el mundo, mientras que las variedades asiáticas están más localizadas en Asia.

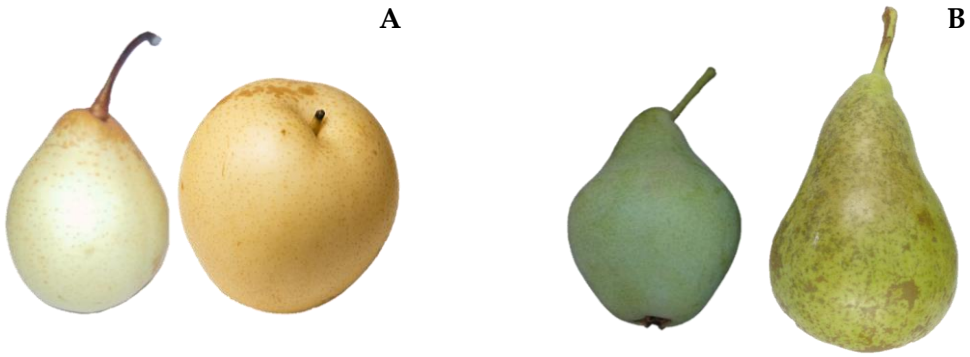

Figura 2. Apariencia morfológica de variedades asiáticas (A) y variedades europeas (B).

A pesar de pertenecer al género *Pyrus*, existen grandes diferencias entre la pera europea y la pera asiática. En primer lugar, lo más característico y visual es la morfología, ya que las peras asiáticas son, de manera general, más redondeadas (con una morfología similar a la manzana) mientras que la europea es piriforme. Respecto a la calidad organoléptica, la pera asiática presenta poco aroma y un sabor suave, mientras que las variedades europeas se caracterizan por un fuerte aroma y sabor; además, presentan diferentes texturas, siendo la pera asiática más crujiente que la europea (Wu et al., 2018). Por otro lado, considerando la fisiología de la maduración, la pera europea se considera un fruto climatérico (término que comentaremos más adelante) mientras que en pera asiática existen variedades con diferentes niveles de producción de etileno y que se suelen clasificar como climatéricas y no climatéricas (Itai et al., 2002). Teniendo en cuenta todo esto, podemos comprobar la gran variabilidad genética que existe dentro del género *Pyrus*.

1.2. *Pyrus communis* L.

Pyrus communis es una especie con una gran variedad de cultivares; en la figura 3 podemos ver alguna de las variedades más representativas.

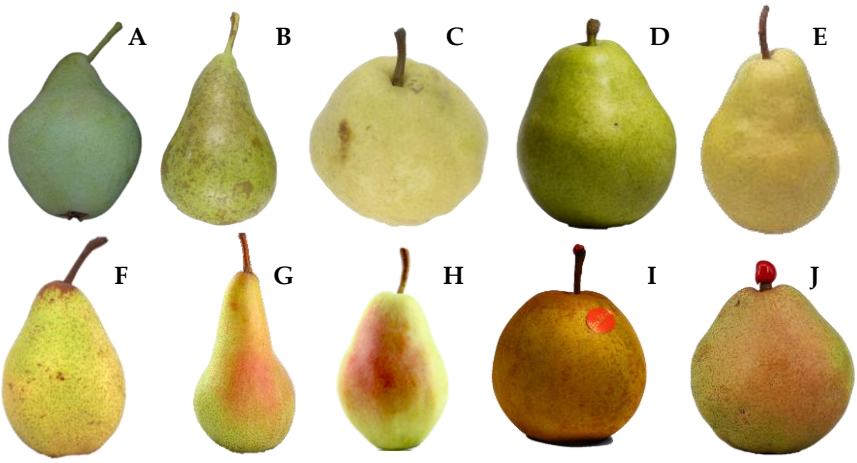

Figura 3. Diferentes variedades del género *Pyrus communis*: Blanquilla (A), Conference (B), Flor d'Hivern (C), d'Anjou (D), Williams (E), Rocha (F), Abate Fetel (G), Devoe (H), Passe Crassane (I) y Comice (J).

En la presente tesis nos hemos centrado en las variedades "Blanquilla", "Conference" y "Flor d'Hivern" ya que están caracterizadas por un patrón de maduración y una susceptibilidad a alteraciones fisiológicas distintos. "Conference" es la variedad con mayor producción en Cataluña (40 %), seguida de "Limonera" (15 %) y "Williams" (14 %), mientras que "Blanquilla" representa el 10 % de la producción total (DARP, 2019b). La pera "Blanquilla", también conocida como pera de agua o pera de Aranjuez (Fig. 3A), es una variedad que se cosecha a principios de agosto, cuando está a unos valores de firmeza entre 54 y 64 N (Llorens et al., 2013). Esta variedad produce etileno nada más es cosechada y es muy sensible al manejo poscosecha y a la aparición de fisiopatías (p.ej: escaldado superficial) durante el almacenamiento en frío. Su periodo de conservación puede ir desde los 3 hasta los 8 meses dependiendo de las condiciones de conservación. "Conference" (Fig. 3B), por otro lado, es una variedad que se cosecha durante la segunda quincena de agosto, con unos valores de firmeza iguales a los de "Blanquilla" (Llorens et al., 2013). La principal característica de esta variedad es la presencia de *russeting*, unas manchas marrones de tacto áspero y que, a pesar de que en algunas variedades se puede considerar un defecto, en otras es característico de la variedad y se llega incluso a inducir mediante aplicaciones de cobre. A diferencia de la pera "Blanquilla", esta variedad no produce etileno a no ser que sea sometida a un periodo a bajas temperaturas. Se considera de sensibilidad baja al escaldado superficial si bien es muy común la aparición de una fisiopatía denominada "mancha negra" y de características similares al escaldado. "Flor

d'Hivern" (Fig. 3C) es una variedad local que se cosecha a valores de firmeza inferiores a los de las variedades anteriores (44 – 59 N) (Llorens et al., 2013) y los niveles de producción de etileno son basales. Su periodo de conservación es también bastante inferior (1 – 4 meses) y es una variedad altamente sensible al escaldado superficial (Recasens y Soria, 2013).

2. Frutos climatéricos y frutos no climatéricos

La primera vez que se habló de la clasificación de los frutos en climatéricos y no climatéricos fue a principios del siglo XX (Kidd y West, 1930) y se hizo en función del comportamiento que mostraban respecto a la tasa respiratoria y la producción de etileno durante la maduración. Desde entonces se asumió que los frutos climatéricos eran aquellos que mostraban un pico en la producción de etileno y CO_2 justo al comienzo de la maduración mientras que los no climatéricos no mostraban dichos picos (Biale, 1964). Además, estos picos van acompañados de unos cambios en la coloración, textura, aroma y otros atributos de los frutos, típicos de la maduración (Fig. 4) (Hiwasa-Tanase y Ezura, 2014). Otra característica que se utiliza para diferenciar a los frutos climatéricos de los no climatéricos es la capacidad para madurar una vez han sido separados de la planta madre. Los primeros son capaces de madurar fuera de árbol si son cosechados en un estado óptimo de madurez, mientras que los no climatéricos necesitan estar unidos al árbol para completar su proceso de maduración (Paul et al., 2012).

El tratamiento con propileno (compuesto análogo del etileno) también ha servido para diferenciar entre frutos climatéricos y no climatéricos. Este tratamiento en los primeros induce la producción de etileno mientras que en los no climatéricos (fresa, cítricos, uva, cereza, etc.) e incluso en determinados cultivares de pera asiática (Downs et al., 1991; Yamane et al., 2007), no. Sin embargo, la existencia de algunos frutos considerados no climatéricos que muestran un pico, aunque bajo, en la producción de etileno al inicio de la maduración (Chervin et al., 2004; Giné-Bordonaba et al., 2017) o en los cuales se ha demostrado que el etileno tiene un papel en la regulación de procesos relacionados con la maduración (Alonso et al., 1995), hace que esta clasificación, a pesar que se siga utilizando, se quede un poco obsoleta.

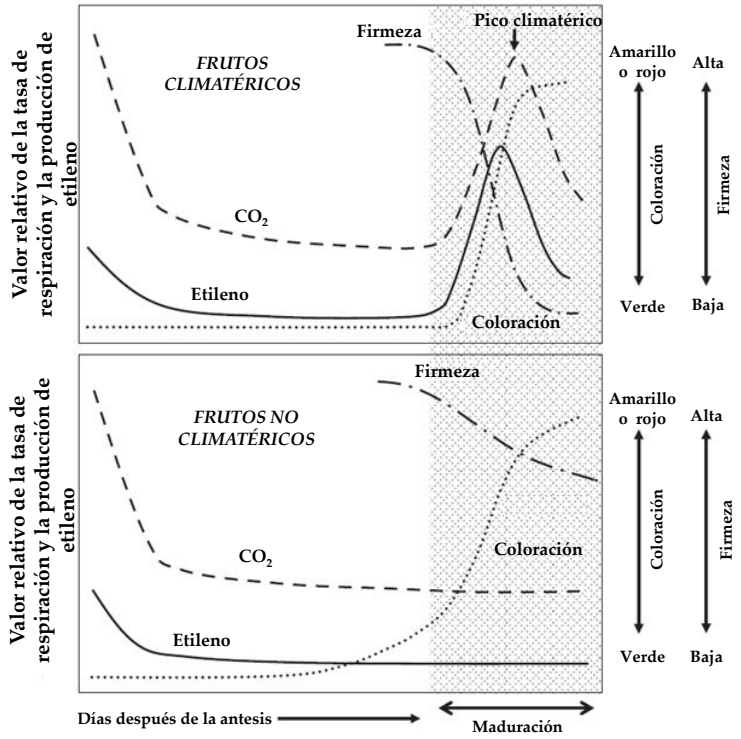

Figura 4. Patrones de maduración en frutos climatéricos y no climatéricos. Figura adaptada de Hiwasa-Tanase y Ezura, 2014.

Ya en 1972, McMurchie et al. definieron dos sistemas de producción de etileno que denominaron como sistema 1 y sistema 2. El sistema 1 se caracteriza por producir niveles basales de etileno, estar presente durante las primeras etapas del crecimiento y desarrollo del fruto y regularse de una manera auto-inhibitoria; por otro lado, el sistema 2 está presente durante la senescencia foliar y la maduración del fruto, es responsable del pico de etileno observado al inicio de la maduración en frutos climatéricos y se regula auto-catalíticamente. Es por esto, que se asume que los frutos climatéricos presentan ambos sistemas de producción de etileno, mientras que los no climatéricos siempren están en el sistema 1, no tienen la capacidad de inducir el sistema 2, y por ello no muestran el pico en la producción de etileno (Fig. 5).

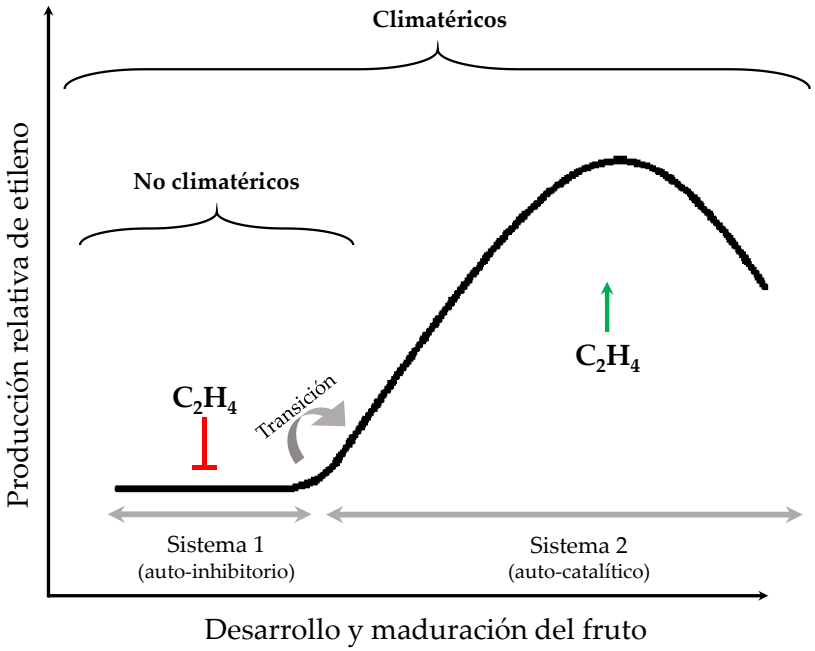

Figura 5. Esquema de los sistemas 1 y 2 de producción de etileno. Figura adaptada de Liu et al. (2015).

3. Etileno: la hormona de la maduración

Las hormonas vegetales son un grupo de sustancias orgánicas que participan en una gran variedad de procesos fisiológicos, desde la diferenciación celular hasta la senescencia del fruto (Davies, 2010). Ya en el siglo XIX se demostró la existencia de unas sustancias capaces de viajar desde su lugar de síntesis hasta el lugar de acción para regular diferentes procesos del crecimiento de la planta (Darwin, 1880). Actualmente se conocen 5 grandes grupos de hormonas: ácido abscísico, auxinas, giberelinas, citoquininas y etileno, siendo esta última la principal hormona relacionada con el crecimiento y la maduración de los frutos climatéricos (Srivastava, 2002a). A pesar de que el etileno ya era utilizado por los egipcios para promover la maduración de higos (Galil, 1968), no fue hasta a principios del siglo XX cuando Neljubov (1901) demostró que este gas tenía un efecto biológico induciendo lo que se denominó la "triple respuesta" (reducción del crecimiento en longitud, incremento del diámetro del hipocótilo y pérdida de la verticalidad) en plántulas de guisante.

3.1. Biosíntesis del etileno

El etileno es una pequeña molécula formada por dos átomos de carbono y cuatro de hidrógeno (Fig. 6), biológicamente activa a concentraciones muy bajas (0,01 – 1 ppm) (Chang, 2016). El etileno se sintetiza a partir de la S-adenosil-L-metionina (SAM), el cual es un compuesto sintetizado a partir del aminoácido metionina en el Ciclo de Yang (Fig. 7). La SAM se transforma en ácido-1-aminociclopropano-1-carboxílico (ACC) gracias a la acción de la enzima ACC sintasa (ACS). En este paso también se produce el compuesto 5-metiltioadenosina (MTA), el cual se recicla a través del Ciclo de Yang para así mantener unos niveles constantes de metionina (Yoon, 2015). El último paso en la síntesis de etileno es la oxidación del ACC por la enzima ACC oxidasa (ACO), produciéndose también CO_2 y HCN. La enzima ACS se suele considerar el paso limitante en la producción de etileno (Yang y Hoffman, 1984), aunque estudios recientes resaltan la importancia de la ACO en aquellos procesos con una alta producción de etileno (Vanderstraeten y van Der Straeten, 2017).

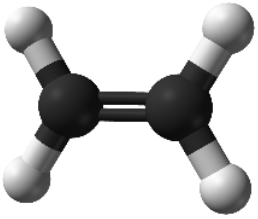

Figura 6. Estructura molecular del etileno.

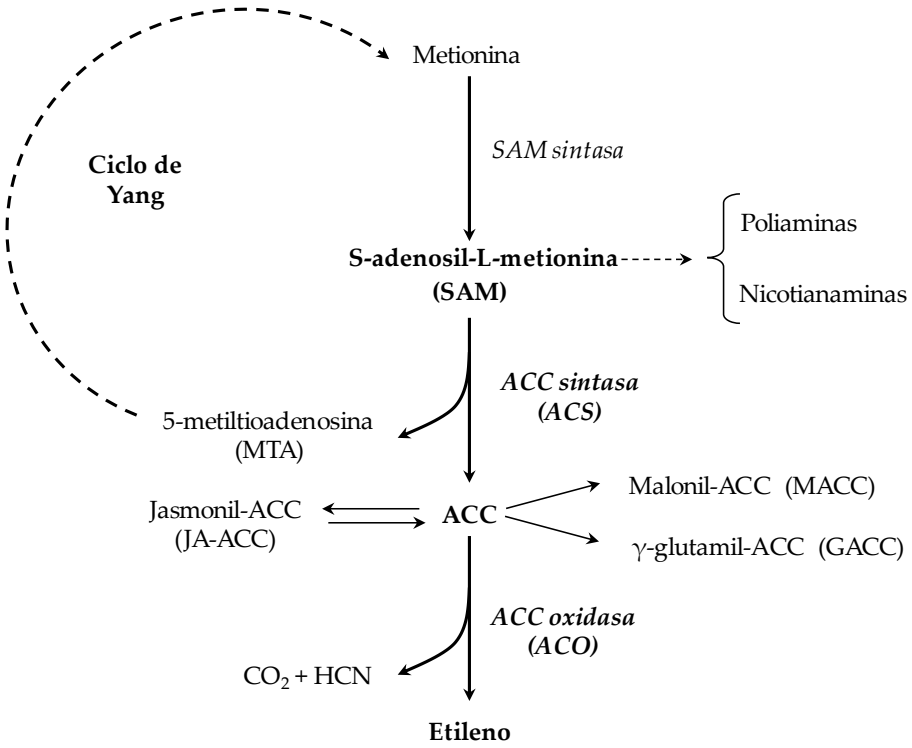

Figura 7. Ruta de biosíntesis del etileno y metabolismo de sus precursores. Figura adaptada de Booker y DeLong (2015).

Cabe destacar la importancia del *pool* de ACC existente en la planta ya que, además de ser el precursor directo del etileno, también se puede conjugar y formar malonil-ACC (MACC), γ-glutamil-ACC (GACC) y también se puede conjugar con otras hormonas, como el ácido jasmónico, reduciéndose así la cantidad de ACC disponible para ser oxidado a etileno.

3.2. Regulación de la ruta de síntesis de etileno

La síntesis del etileno es una ruta que está altamente regulada por una gran variedad de factores internos (germinación, senescencia, abscisión del fruto…) y externos (heridas, inundación, ataque de patógenos, aplicación de químicos…). Es por ello, que se le dé bastante importancia a la regulación transcripcional y postraduccional de las principales enzimas de la ruta (ACS y ACO). Además de estos

factores de regulación, la biosíntesis del etileno resalta por su capacidad para autoinhibirse o autoinducirse.

3.2.1. Regulación de ACC sintasa

La ACC sintasa está formada por una familia multigénica y, a pesar de que la enzima purificada es activa en su forma monomérica, en tejidos vegetales se encuentra formando dímeros, y ésta es la forma activa de la proteína (Argueso et al., 2007; Pech et al., 2010). En un estudio reciente del genoma de la pera asiática (*Pyrus ussuriensis*) se ha revelado la existencia de 13 genes de la enzima ACS (*PuACS1* → *PuACS12*). Los patrones de expresión analizados en diferentes tejidos, indican que estos genes se encuentran diferencialmente regulados; por ejemplo: *PuACS1* y *PuACS4* están altamente expresados en frutos y no en raíces, tallo, hojas y flores, mientras que *PuACS7* y *PuACS9* no se expresan en el fruto pero sí en el resto de tejidos. Además, todos estos genes también responden de manera diferente a tratamientos con etileno y con 1-metilciclopropeno (1-MCP; antagonista del etileno) (Yuan et al., 2019). Por otro lado, gracias al estudio del genoma de la variedad "Bartlett" (Chagné et al., 2014), se han identificado 9 genes que codifican para la ACS (http://www.rosaceae.org/species/pyrus/pyrus_communis/genome_v1.0) en la especie *Pyrus communis* que tienen diferentes patrones de acumulación de transcritos durante la maduración, la conservación en frío o como respuesta a tratamientos con 1-MCP (El-Sharkawy et al., 2004b; Villalobos-Acuña et al., 2011; Xie et al., 2014). También pasa algo similar en tomate (*Lycopersicon esculentum*) y manzana (*Malus domestica*), donde las isoformas del gen de la ACS muestran diferentes patrones de expresión durante la maduración (Barry et al., 2000; Li et al., 2013) y, en el caso del tomate, también tienen diferentes respuestas al etileno y al 1-MCP (Nakatsuka et al., 1998).

Además de la regulación transcripcional, la ACC sintasa también se regula a nivel postraduccional mediante fosforilación de la región C-terminal, lo cual no altera su actividad enzimática pero sí aumenta su estabilidad y altera el recambio proteico (*protein turnover*) (Pech et al., 2010; Wang et al., 2002). El estudio llevado a cabo por Tatsuki y Mori (2001) en plantas de tomate demostró que no todas las isoformas del gen sufren la misma modificación postraduccional, ya que hay isoformas que no contienen el aminoácido que se fosforila y, por lo tanto, no sufren dicha modificación.

3.2.2. Regulación de ACC oxidasa

Al igual que la ACS, la enzima ACC oxidasa también forma parte de una familia multigénica. La caracterización de esta enzima ha sido muy difícil, de hecho, durante mucho tiempo se la conocía simplemente como "enzima formadora de etileno (EFE)". Gran parte de la problemática de su purificación se debe a que desde un principio se asumió que era una proteína unida a la membrana celular (Apelbaum et al., 1981) en lugar de considerarse soluble, como se descubrió años más tarde (John, 1991). A pesar de estos estudios, se sigue discutiendo bastante sobre la localización subcelular de esta enzima. En el trabajo de Yuan et al. (2019) mencionado anteriormente, se caracterizaron 11 isogenes de ACO en *P. ussuriensis*, los cuales muestran patrones de expresión muy diferentes en función del tejido vegetal y del estadio fenológico en el que se encuentre la planta (Lin et al., 2009). Por ejemplo, *PuACO2* se expresa en mayor medida en frutos, mientras que *PuACO4* y *PuACO5* se expresan en todos los tejidos analizados salvo en frutos. *PuACO6* no se expresó en ninguno de los tejidos analizados. En este mismo estudio, también se demostró las diferentes respuestas de estos genes a tratamientos con etileno y con 1-MCP.

A pesar de que se ha especulado bastante sobre la actividad proteasa de esta enzima y las posibles interacciones entre las ACC oxidasas, actualmente solo se han descrito dos modificaciones a nivel postraduccional de dicha proteína. Se ha visto que *AtACO1* puede sufrir una glutationalización en plantas de Arabidopsis, (Datta et al., 2015) y, en tomate, *LeACO1* y *LeACO2* puede ser persulfuradas (Jia et al., 2018).

3.3. Procesos en los que interviene la hormona etileno

El etileno es una hormona que interviene en una inmensa variedad de procesos relacionados con el desarrollo de la planta, aunque bien es verdad que su papel más relevante es el de la regulación de la maduración en frutos climatéricos. A continuación, se detallan algunos procesos en los que actúa esta hormona.

- Desarrollo de la semilla: periodo de latencia y germinación

La latencia es una etapa del desarrollo de la semilla cuyo objetivo principal es evitar que germine en un entorno que no sea apto para su desarrollo (Considine y Considine, 2016). Es un periodo que está controlado por muchos factores exógenos (luz, temperatura…) y también endógenos (hormonas, moléculas de señalización…). Se sabe que el ácido abscísico induce el estado de latencia y que las giberelinas son

capaces de interrumpirlo y promover la germinación (Finkelstein et al., 2008); sin embargo, otros estudios también relacionan al etileno con la inducción de la latencia o la germinación, dependiendo de la especie (Matilla y Matilla-Vázquez, 2008). Actualmente sigue existiendo controversia sobre si el etileno es una hormona necesaria para que se produzca la germinación o si, por el contrario, es un producto de esta (Matilla, 2000).

- Crecimiento y desarrollo de la planta

Diferentes estudios han detallado la implicación del etileno durante el desarrollo vegetativo de la planta: formación de raíces y gancho apical, desarrollo de meristemos y crecimiento del hipocótilo entre otros (Dugardeyn y Van Der Straeten, 2008). Además, también se le relaciona con la inducción o el retraso de transición floral (transición de un crecimiento vegetativo a otro reproductivo) y con la determinación del sexo en flores de plantas de la familia de las Cucurbitáceas (Vandenbussche y Van Der Straeten, 2012).

- Senescencia foliar

La senescencia es la última fase del desarrollo de la planta, cuando se produce una reorganización de los nutrientes desde los tejidos fotosintéticos y las flores hacia la formación de frutos y semillas (Graham et al., 2012). Es cierto que el etileno tiene un papel bastante bien definido en la promoción de la senescencia foliar, pero cabe destacar que no realiza él solo esta función sino que actúa conjuntamente con otras hormonas que o bien retrasan la senescencia foliar (giberelinas, auxinas y citoquininas) o bien la promueven (ácido abscísico, brasinoesteroides y los ácidos jasmónico y salicílico) (Schippers et al., 2007).

- Maduración del fruto

A pesar de que la regulación de la maduración por etileno está principalmente relacionada con la de los frutos climatéricos, diversos estudios demuestran que también tiene un papel, aunque no principal, en frutos no climatéricos. Por ejemplo, en un estudio llevado a cabo por Chervin et al. (2004) en uva, considerada no climatérica, se observó un aumento en la producción de etileno justo antes del envero (punto que indica el inicio de la maduración), el cual era necesario para el aumento del diámetro y la acumulación de antocianinas. Algo parecido se ha descrito en limón,

donde el etileno parece ser importante para el cambio de color en la piel, la degradación de clorofilas y la síntesis de carotenoides (Goldschmidt, 1998), y también en fresa y cereza, donde se puede observar un aumento de la producción de etileno y de la tasa respiratoria cuando la fruta está virando de color (Giné-Bordonaba et al., 2017; Iannetta et al., 2006).

En varios trabajos realizados con mutantes de fruta, se ha podido comprobar que el etileno está íntimamente relacionado con el desarrollo de aromas. En estudios con tomate, manzana y melón con el gen de la ACS o la ACO silenciados, se vio una reducción en la producción de ésteres, mientras que la producción de otros compuestos, como alcoholes y aldehídos, no se vio especialmente afectada (Baldwin et al., 2000; Dandekar et al., 2004; Flores et al., 2002). Además del aroma, el etileno también interviene en el desarrollo del color. En tomates con la ruta de síntesis del etileno alterada, se ha visto una reducción en la degradación de la clorofila y un bajo contenido en los pigmentos típicos de la coloración roja (licopenos y carotenoides) (Alexander y Grierson, 2002). Defilippi et al. (2004) y sus estudios en manzana demostraron que la acumulación de azúcares y la degradación de ácidos característicos de la maduración se ven afectadas en manzanas con una baja producción de etileno. Sin embargo, respecto a la pérdida de firmeza, existe cierta controversia ya que se ha visto que puede ser etileno dependiente e independiente. Dandekar et al. (2004) mostró que la textura en manzana estaba claramente regulada por el etileno, pero otro trabajo llevado a cabo en melones tratados con 1-MCP demostró que había un cierto retraso en la pérdida de firmeza, pero que esta sí que se producía (Ergun et al., 2005). En tomate también se ha demostrado que la pérdida de firmeza no está totalmente inhibida en mutantes reprimidos para la síntesis de etileno, sólo retrasada (Murray et al., 1993); sin embargo, algunos estudios confirman que una de las enzimas relacionadas con la pérdida de firmeza, la poligalacturonasa, es etileno dependiente (Alexander y Grierson, 2002) y mutantes "nonripening" (*nor*) y "ripening-inhibitor" (*rin*) de tomate muestran una baja actividad de esta enzima (Baldwin et al., 2000).

INTRODUCCIÓN

- Parte I: Maduración de la pera -

Los consumidores somos cada vez más exigentes y queremos fruta de alta calidad organoléptica, que aguante bien en casa y sin tratamientos en campo ni poscosecha; sin embargo, teniendo en cuenta el punto de vista del agricultor, el cual quiere una alta producción y distribuir su producto a lo largo del año, hay que encontrar el balance entre calidad y eficacia del cultivo. De aquí nace la importancia de conocer a fondo la fisiología de la maduración del fruto que, en el caso concreto de la pera, sigue siendo una gran desconocida.

El escaldado superficial, del cual hablaremos en detalle más adelante, es una alteración fisiológica que afecta principalmente a peras y manzanas y aparece tras largos periodos de almacenamiento en frío, por lo que la mayoría de investigaciones están orientadas a estudiar este desorden después del almacenamiento. Sin embargo, existen pocos estudios que relacionen lo ocurrido durante el desarrollo y maduración del fruto con la incidencia de esta alteración y nosotros creemos que es de vital importancia conocer la fisiología del desarrollo del fruto para entender mejor el porqué de la aparición del escaldado superficial.

1. Morfología y fisiología del desarrollo

El fruto tiene como origen el ovario de la flor, el cual después de la polinización experimenta una gran variedad de cambios fisiológicos, moleculares y bioquímicos que hacen posible su evolución hacia una estructura mucho más compleja. Desde la fecundación, el ovario se expande y el crecimiento del fruto se suele dividir en fases cuyo número varía dependiendo del fruto. En el caso de pomáceas, el crecimiento del fruto sigue una curva sigmoidea constituida por 3 fases, las cuales se caracterizan por: (I) crecimiento lento debido a división celular, (II) fase exponencial de crecimiento debido a expansión celular y (III) fase estacionaria correspondiente a la maduración del fruto (Fig. 8) (Jackson, 2003). La primera fase comienza justo después de la polinización y dura unas semanas, dependiendo de la duración del desarrollo del fruto (Coombe, 1976); después, durante la fase II, las divisiones celulares se ralentizan y predomina el incremento del tamaño de las células de manera exponencial. Por último, a pesar de que las células se siguen dividiendo y expandiendo, se llega a una fase de lento crecimiento (en algunos casos incluso estacionario) hasta el momento de cosecha.

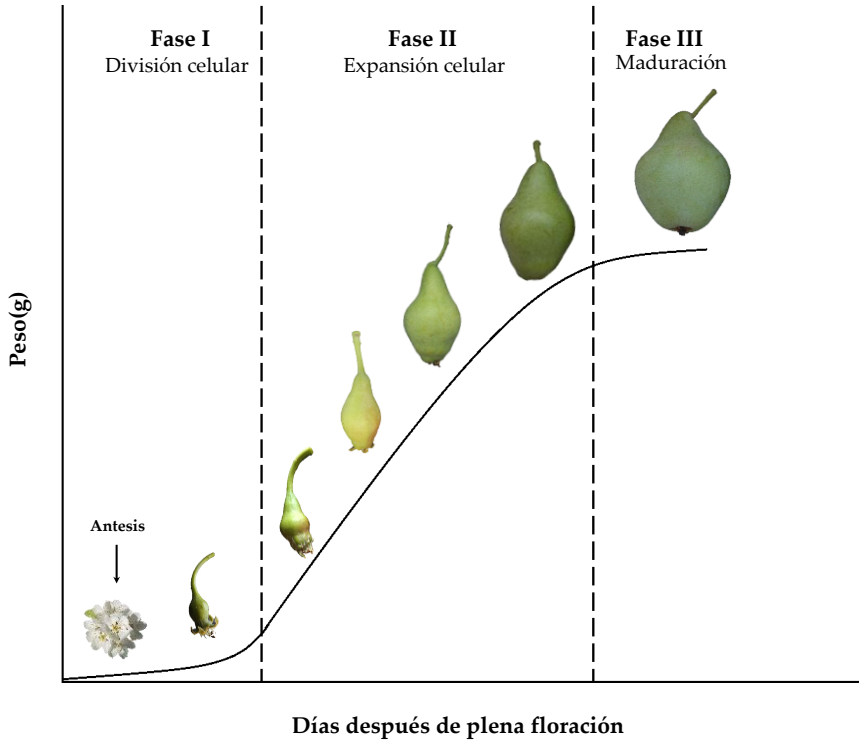

Figura 8. Evolución de pera "Blanquilla" durante las tres fases de crecimiento.

2. Principales cambios durante el desarrollo y la maduración del fruto

2.1. Peso y diámetro

El aumento del tamaño es el primer cambio que se nos viene a la cabeza si pensamos en el desarrollo de un fruto. Durante el paso de flor a fruto, la pera puede aumentar su peso entre 200 – 600 veces y entre 6 – 10 veces su diámetro (Tabla 1). Este incremento se debe principalmente al aumento del número y tamaño de células y a la acumulación en ellas de metabolitos básicos.

Tabla 1. Evolución del peso y diámetro (datos propios) de las variedades "Blanquilla" y "Conference". [1]DDPF = Días después de plena floración. [2]DOC = Día óptimo de cosecha.

	Peso (g)		Diámetro (mm)	
DDPF[1]	"Blanquilla"	"Conference"	"Blanquilla"	"Conference"
10	0,56	0,49	8,84	7,43
30	3,88	3,14	8,50	14,60
50	11,11	14,16	24,11	25,86
70	26,05	42,87	32,87	38,98
90	52,27	96,24	42,41	53,77
110	86,62	146,82	50,96	61,52
DOC[2]	111,73	217,49	54,20	70,52

2.2. Textura

La firmeza es uno de los atributos más característicos de la textura y uno de los parámetros más importantes a la hora de predecir la fecha de cosecha de un fruto. Los principales compuestos de la pared celular del tomate, y de otros muchos frutos, son la celulosa, la hemicelulosa y la pectina, siendo ésta última la más asociada a los cambios producidos durante la maduración (D. Wang et al., 2018). La degradación de estos compuestos por diferentes enzimas (celulasas, endo-poligalacturonasa, pectinmetilestarasa, pectato liasas, etc.) produce el ablandamiento del fruto durante la maduración. En pera europea, se ha visto que las endo-poligalacturonasas (endo-PGs) y las enzimas despolimerizadoras de hemicelulosa son las principales responsables de la textura en el cultivar "La France"(Hiwasa et al., 2004). Resultados similares se encuentran en el estudio llevado a cabo por Song et al. (2016) con otras tres especies de *P. communis*, donde se recalca la importancia de las poligalacturonasas en la pérdida de firmeza durante la maduración del fruto y, además, se demuestra que las diferencias observadas en la expresión de los genes de PGs podrían explicar los diferentes patrones de ablandamiento del fruto.

2.3. Color

El cambio de color que experimentan los frutos durante la maduración, y el cual se considera un buen indicador de la madurez en algunos de ellos, implica la degradación de clorofilas y la acumulación de otros pigmentos. Los principales

pigmentos que podemos encontrar en plantas son: antocianinas, carotenoides y betalaínas; siendo estos últimos un grupo muy restringido ya que sólo se encuentra en el orden de las Caryophyllales. En general, la pera europea presenta un color verde en su estadio inmaduro, y algunas variedades viran a amarillo durante la maduración. En un estudio reciente llevado a cabo con las variedades "La France" (permanece verde) y "Gorham" (vira de color), se ha visto que el color verde se debe a un alto contenido en clorofilas pero que el color amarillo se debe al desenmascaramiento de los carotenoides (producido por la degradación de las clorofilas) más que a una acumulación de éstos (Charoenchongsuk et al., 2018). Además, también nos podemos encontrar con peras rojas (cultivar "Starkrimson" y "Red Bartlett"), cuya coloración se debe principalmente a la acumulación de antocianinas (Z. Wang et al., 2017). Sin embargo, algunas variedades que no son rojas en su totalidad, sí presentan tonalidades rojas durante su desarrollo, observándose un pico en acumulación de antocianinas (Steyn et al., 2004) (Fig.9). Un factor clave en la acumulación y degradación de las antocianinas es la exposición a la luz solar, ya que ésta regula las rutas de síntesis y degradación (Chalker-Scott, 1999) y es por ello que dicha coloración suela aparecer en la zona del fruto que está expuesta a la luz solar.

Figura 9. Acumulación de antocianinas en pera "Conference" a 50 días después de plena floración (50 DDPF).

Teniendo en cuenta todo esto, cabría destacar la importancia del índice de diferencia de absorbancia: IAD. Este es un parámetro no destructivo desarrollado inicialmente para medir la madurez en kiwi (Cantin et al. 2011) y se calcula como la diferencia de absorbancias entre 670 nm (cerca del pico de absorción de la clorofila *a*) y 720 nm (interferencias de fondo). Actualmente, el IAD se utiliza bastante para conocer el estado de madurez de melocotones y nectarinas (Ziosi et al., 2008), aunque también se han descrito valores de cosecha para la variedad "Gala" en manzana (Costamagna et al., 2013) y la variedad de pera "Abbate Fetel" (Vidoni et al., 2015).

2.4. Aroma

El desarrollo de aromas agradables durante la maduración del fruto es de vital importancia ya que implica la aceptabilidad o no por parte del consumidor, y también es un gran atrayente para diversos animales (polinizadores o encargados de dispersar las semillas). También resulta interesante mencionar el flavor, ya que engloba tanto al aroma (considerado como el olor percibido por los orificios nasales) como el sabor (percibido por la lengua). El flavor es percibido por ambos sentidos durante la masticación y es el responsable de la calidad organoléptica del fruto. El aroma de un fruto se debe a una infinidad de compuestos volátiles que, incluso a concentraciones muy bajas, pueden ser los responsables del olor característico de un fruto. Los principales compuestos volátiles pueden ser: ésteres, alcoholes, aldehídos, cetonas, lactonas, terpenoides y apocarotenoides (El Hadi et al., 2013), siendo los primeros los mayoritarios en pera europea (representando entre el 60 y el 99 % de los volátiles emitidos (Rapparini y Predieri, 2003)). Dentro de los ésteres, podemos destacar el acetato de hexilo y acetato de butilo, que son los ésteres mayoritarios en una gran variedad de peras (Suwanagul y Richardson, 1998). Además de estos dos ésteres, también son de gran importancia los diversos ésteres del ácido decadienoico, tal y como se demuestra en diversos estudios con la variedad "Bartlett" (Creveling y Jennings, 1970; Heinz y Jennings, 1966; Jennings et al., 1964).

Los compuestos volátiles se sintetizan principalmente: I) a partir de aminoácidos, II) a partir de lípidos y III) a partir de carbohidratos. La primera ruta de síntesis es muy poco usual en pera, siendo la gran mayoría de los volátiles identificados sintetizados a partir de lípidos (principalmente de los ácidos linoleico y linolénico) y mediante dos vías diferentes: β-oxidación y a través de la enzima lipoxigenasa (LOX). La ruta de la β-oxidación (eliminación sucesiva de dos unidades de carbono, en forma de acetil-CoA, del ácido graso principal) es la encargada de la síntesis de los volátiles primarios (aquellos que son emitidos por el fruto intacto) y es considerada la ruta más

importante en pera (Rapparini y Predieri, 2003). Por otro lado, la vía de la LOX es responsable de la síntesis de una amplia gama de compuestos secundarios (emitidos cuando el tejido se rompe, se corta o incluso se mastica (Rapparini y Predieri, 2003) y que, por lo tanto, es la principal vía para el desarrollo del flavor del fruto). Sin embargo, se ha visto que la vía de la LOX también está activada durante la maduración y senescencia de los frutos (El Hadi et al., 2013). Los principales productos de ambas vías son aldehídos, los cuales pasan a formar alcoholes gracias a la acción de alcohol deshidrogenasas (ADH) y éstos son transformados a ésteres por la acción de alcohol aciltransferasas (AAT) (Rapparini y Predieri, 2003).
Los volátiles sintetizados a partir de carbohidratos son principalmente terpenos y furanonas. Los primeros provienen de la ruta de síntesis del ácido mevalónico y en nuestro caso destacaremos el α-farneseno, el cual es un sesquiterpeno que a pesar de no tener características aromáticas es un compuesto fundamental en la aparición del escaldado superficial, del cual hablaremos detalladamente en la parte II de la introducción (Rapparini y Predieri, 2003).

Las principales enzimas involucradas en la síntesis de compuestos volátiles (LOX, ADH y AAT) están reguladas por etileno, y además se ha visto que el pico climatérico de etileno y CO_2 precede al pico de ésteres (Rapparini y Predieri, 2003; Zhu et al., 2005). Los aromas, además de ser considerados como algo positivo y característico de los frutos, también pueden ser indicadores de alteraciones fisiológicas, ya que en algunas situaciones de estrés los frutos producen olores desagradables. Un claro ejemplo es la producción de etanol y acetaldehído como resultado de un metabolismo fermentativo durante largos periodos o bajo ciertas condiciones de almacenamiento (Richardson y Kosittrakun, 1995; Saquet, 2016); por lo tanto, el análisis de volátiles puede ser una herramienta útil para monitorizar diferentes procesos fisiológicos de la planta como la maduración, la senescencia o la aparición de desórdenes internos.

2.5. Sabor

Los principales componentes que determinan el flavor de un fruto son: azúcares, ácidos y volátiles. De estos últimos ya hemos hablado detalladamente en el apartado anterior por lo que ahora nos centraremos en los azúcares y los ácidos.
De los cinco sabores descritos, el dulce es el que más caracteriza a un fruto y está definido por la cantidad de azúcares presentes en el fruto y por el ratio de éstos (Osorio y Fernie, 2014). Los azúcares más comunes son fructosa, glucosa y sacarosa (Tucker, 1993), destacando también el polialcohol sorbitol (Eccher Zerbini, 2002). En

pera, la fructosa es el azúcar mayoritario, estando en concentraciones 2 veces mayores que la glucosa y entre 5 y 10 veces mayores que la sacarosa (Yim y Nam, 2016). Diferentes estudios demuestran que el contenido en azúcares aumenta durante el desarrollo y la maduración de los frutos (Amorós et al., 2003; Giné-Bordonaba et al., 2019, 2017) y esto puede deberse principalmente a una mayor importación por parte de la planta madre mientras está madurando en el árbol y a la degradación del almidón una vez que el fruto ha sido cosechado (Tucker, 1993). El almidón es el principal reservorio de energía de un fruto, por lo que conforme avanza la maduración se va degradando gracias a la acción enzimática de amilasas y fosforilasas transformándose en azúcares simples, como glucosa y fructosa (Tucker, 1993). Durante muchos años se ha asumido que los azúcares están involucrados en el metabolismo basal de las células; sin embargo, estudios recientes apuntan a que intervienen también en otros muchos procesos. Son sustratos respiratorios, metabolitos intermedios en muchos procesos bioquímicos, tienen función protectora frente a situaciones de estrés (actuando como osmoprotectores o como moléculas de señalización), y también forman parte de la regulación del ciclo celular, diferenciación celular, floración, formación del fruto y senescencia, entre otros (Ciereszko, 2018; Eveland y Jackson, 2012; Lastdrager et al., 2014).

El contenido en ácidos, en menor medida, también es un buen indicador del sabor y de la calidad nutricional (Sha et al., 2011) y, además, el ratio azúcares/acidez se suele utilizar como índice de madurez para determinar el momento de cosecha. Aunque la composición de ácidos es mucho más variable que la de azúcares, se sabe que el ácido mayoritario en pera es el málico, seguido del ácido cítrico (Eccher Zerbini, 2002). Además de conferir sabor, el ácido málico es un intermediario del ciclo de Krebs, un importante reservorio de carbono y un regulador de pH (Fernie y Martinoia, 2009).

3. Maduración de frutos climatéricos

3.1. Peras de verano vs. Peras de invierno

Las peras europeas se engloban dentro de los frutos climatéricos, los cuales son capaces de madurar una vez han sido cosechados; sin embargo, algunas variedades de pera, a pesar de ser cosechadas en su punto óptimo, no son capaces de madurar a no ser que se les aplique un tratamiento con etileno o se sometan a un periodo de almacenamiento a bajas temperaturas. Es por ello que las peras europeas se clasifican en peras de verano y peras de invierno. Las primeras son aquellas que no requieren

un tratamiento con etileno o con bajas temperaturas para madurar después de la cosecha o, si lo requieren, es muy corto. En cambio, las peras de invierno sí requieren al menos uno de estos dos tratamientos para poder madurar y, dentro de estas últimas, nos podemos encontrar con variedades de pera que necesitan en torno a un mes de frío para producir etileno mientras otras requieren tres o incluso más meses. En la Tabla 2 se muestran algunas variedades de pera europea y el tiempo necesario de almacenamiento en frío para su correcta maduración.

Tabla 2. Variedades de pera europea y tiempo necesario (en días) para inducir la producción de etileno.

Variedad	Tiempo de almacenamiento en frío necesario (días)	Bibliografía
"Blanquilla"	0	Lindo-García et al. (2019)
"Conference"	15	Lindo-García et al. (2020)
"Bartlett"	14	Agar et al. (2000)
"Rocha"	0	Saquet y Almeida (2017)
"Beurré d´Anjou"	60 - 150	Larrigaudière et al. (2016) y Sugar y Einhorn (2011)
"Packham Triumph"	15	Larrigaudière et al. (2016)
"Bosc"	< 14	Elgar et al. (1997)
"Abate Fetel"	20	Zucoloto et al. (2016)
"Doyenné du Comice"	25 - 31	Sugar y Basile (2006)
"La France"	21	Hiwasa et al. (2003)
"Passe Crassane"	80	El-Sharkawy et al. (2004a)

3.2. *Cross-talk* hormonal durante la maduración

A pesar de que el etileno se ha considerado durante años como la hormona principal del desarrollo y la maduración de los frutos climatéricos, cada vez más estudios apuntan a un efecto coordinado entre las diferentes hormonas vegetales (comúnmente llamado *cross-talk*) más que a la acción de una única hormona (McAtee

et al., 2013; Munné-Bosch y Müller, 2013). A continuación, haremos una breve descripción de las principales hormonas.

- Auxinas. La principal auxina es el ácido 3-indolacético (IAA), el cual es sintetizado a partir del aminoácido triptófano (Santner et al., 2009). Participa en diferentes eventos como: división y elongación celular, respuestas trópicas a la luz y a la gravedad, senescencia foliar, floración, formación y crecimiento del fruto, etc. (Davies, 2010). Se ha visto que para la formación y posterior crecimiento del fruto es necesaria la interacción y la acción conjunta de auxinas, giberelinas y, en menor parte, citoquininas (Mariotti et al., 2011). Además, existe una interacción entre el etileno y las auxinas ya que estas últimas estimulan la biosíntesis de etileno a través de la inducción de la expresión génica de la ACC sintasa (Bleecker y Kende, 2000).

- Giberelinas (GAs). Se sintetizan a partir del difosfato de geranilgeranilo (Santner et al., 2009) y, a pesar de ser una familia muy numerosa, la giberelina más importante es la giberelina 1 (GA_1) y muchas de las otras GAs son precursoras de esta. La GA_1 está involucrada en procesos como la germinación de la semilla, crecimiento del tallo, floración, etc. (Davies, 2010). Como ya se ha descrito anteriormente, son importantes después de la polinización ya que promueven, junto con las auxinas, la formación del fruto (De Jong et al., 2009). Además, giberelinas y auxinas son las hormonas clave que regulan la división y la elongación celular (Obroucheva, 2014).

- Citoquininas (CKs). Son moléculas derivadas de la base nitrogenada adenina (Santner et al., 2009). A pesar de que el mecanismo de acción y la regulación no ha sido todavía investigado en profundidad, se ha visto que estas hormonas participan en la división celular, senescencia foliar, crecimiento de brotes laterales, apertura de los estomas, etc. (Sakakibara, 2006). La expresión de algunos genes de la ruta de síntesis de las citoquininas está regulada por las mismas citoquininas y también por auxinas y ácido abscísico (Sakakibara, 2006); además de estas hormonas, también se ha visto que las citoquininas interactúan con el etileno, promoviendo una maduración temprana (Iqbal et al., 2017).

- Ácido abscísico (ABA). Es un sesquiterpeno que se sintetiza a partir del isopentenil difosfato (punto de partida para la síntesis de terpenoides). Existen dos rutas de síntesis: a partir del farnesil difosfato o a partir de carotenoides C_{40} (p. ej: violaxantina), siendo esta última la más común en plantas superiores (Srivastava, 2002b). Actualmente se sabe que no es la hormona principal involucrada en la

abscisión, pero su nombre se debe a que en la segunda mitad del siglo XX se identificó y se purificó como la molécula responsable de la abscisión de flores y frutos de algodón (Davies, 2010; Srivastava, 2002b). A pesar de que se considera la hormona principal de la maduración en frutos no climatéricos (Fuentes et al., 2019; Setha, 2012), hay que resaltar su importancia también en los climatéricos, ya que se ha visto que desempeña funciones bastante importantes como potenciar la biosíntesis de etileno (Mou et al., 2016) y, por tanto, promover la maduración (Zhang et al., 2009). También participa en la respuesta a estrés (Srivastava, 2002b).

A pesar de que las hormonas mencionadas anteriormente, junto con el etileno forman el grupo de hormonas que podemos calificar de "clásicas", también queremos mencionar algunas otras que son consideradas como nuevas familias de hormonas (Munné-Bosch y Müller, 2013).

- Jasmonatos. Están representados por el ácido jasmónico (JA) y su metil éster (MeJA). Son oxilipinas que provienen de los fosfolípidos de membrana, como el ácido linolénico (Avanci et al., 2010; Santner et al., 2009). Junto con el ácido salicílico, tiene un papel fundamental en la respuesta al estrés biótico y abiótico, aunque también participan en procesos como formación de tubérculos, desarrollo de la flor, senescencia…(Davies, 2010).

- Ácido salicílico (SA). Importante molécula de señalización en plantas y para cuya síntesis se han propuesto dos vías: a partir del cinamato (proveniente del aminoácido fenilalanina) o a partir del isocorismato (Chen et al., 2009). Dentro de la respuesta al estrés, cabe destacar que es la responsable de activar la resistencia sistémica adquirida (SAR, de sus siglas en inglés) (Wani et al., 2017).

- Brasinoesteroides (BRs). Hormonas sintetizadas a partir del campesterol, uno de los principales esteroles de plantas. Están implicados en respuestas a estrés ambiental, como pueden ser elevadas o bajas temperaturas, sequía, salinidad, etc. Y también en el desarrollo del tubo polínico, epinastia, alargamiento del tallo… entre otros (Bajguz y Piotrowska-Niczyporuk, 2014). Estas hormonas tienen un papel principal en la maduración de frutos no climatéricos (Fuentes et al., 2019).

En algunas especies como la frambuesa, uva, fresa y cereza se han descrito los perfiles hormonales durante el desarrollo y maduración del fruto (Fig. 10) y las interacciones que se dan entre las diferentes hormonas (Fuentes et al., 2019; Teribia et al., 2016); sin embargo, no disponemos de este tipo de información en peras.

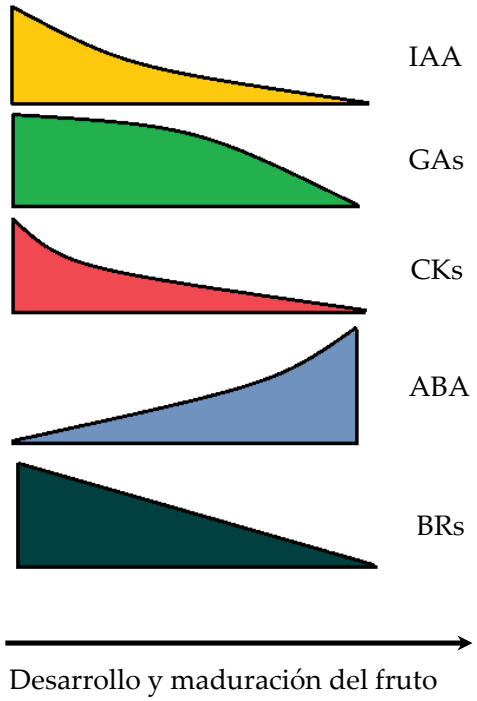

Figura 10. Esquema de la evolución del perfil hormonal en frutos no climatéricos durante el desarrollo y posterior maduración del fruto. Figura basada en las figuras de Fuentes et al. (2019) y Teribia et al. (2016).

INTRODUCCIÓN

- Parte II: El escaldado superficial en pera -

Una vez aclarados algunos conceptos generales y habiendo entrado en detalle en los procesos que ocurren durante el desarrollo y maduración de la pera, ahora profundizaremos lo que pasa durante almacenamiento y qué factores determinan la aparición de alteraciones fisiológicas, con especial énfasis sobre el escaldado superficial.

1. Almacenamiento de la pera

Con el objetivo de conseguir una calidad óptima, la pera se suele cosechar en un estadio preclimatérico y luego se deja madurar en cámara, tratando así de mantener la calidad de la fruta durante el tiempo que sea necesario. La selección de la madurez de la fruta en cosecha depende de para qué fin se destine, ya que una fruta óptima para el consumidor probablemente no sea óptima para un periodo largo de almacenamiento (Saquet, 2019). Para una conservación larga hay que cosechar la fruta a una madurez apropiada, teniendo en cuenta que: una pera cosechada antes de tiempo, aunque pueda aguantar más tiempo en cámara, es de menor tamaño y de peor calidad (déficit de aromas y sabor). Por otro lado, peras cosechadas más tarde, a pesar de tener mejores características organolépticas, tienen una tasa de ablandamiento mayor, son más susceptibles a desórdenes internos y, por lo tanto, la duración del almacenamiento es menor (De Belie et al., 2000). Además, una pera cosechada demasiado madura es más sensible a las alteraciones durante la conservación (Blaszczyk, 2010; Hussein et al., 2020; Silva et al., 2010), siendo el escaldado superficial una de las más problemáticas (Calvo et al., 2015).

1.1. Principales alteraciones fisiológicas durante la conservación

La frigoconservación es algo positivo y necesario para mantener la calidad de la pera; sin embargo, cuando el periodo de almacenamiento es demasiado largo o las condiciones no son las idóneas es probable que aparezcan algunos desórdenes externos y/o internos que empeoran la calidad, haciendo en muchos casos imposible su comercialización. A continuación daremos una pincelada de las principales alteraciones fisiológicas en pera:

- El escaldado superficial: se considera una de las grandes problemáticas del almacenamiento en pera (Fig. 11A). Ahora simplemente lo mencionamos puesto que más adelante hablaremos de él en profundidad.

- El escaldado de senescencia: aparece tras largos periodos de almacenamiento y se caracteriza por el desarrollo de manchas marrones oscuras más intensas que las del escaldado superficial (Fig. 11B). Es una alteración muy típica en la variedad "Williams" (Whitaker et al., 2009).

- Mancha negra: se reconoce como pequeños puntos negros (que pueden llegar a ocupar una superficie considerable de la pera) en la piel de algunas variedades, sobre todo "Conference" y "Beurré d'Anjou". La aparición de esta fisiopatía se asocia principalmente a las bajas temperaturas junto con una presión parcial baja de oxígeno (Mattheis y Rudell, 2011; Rizzolo et al., 2015).

- Pardeamiento interno: se caracteriza por un oscurecimiento inicial del corazón y aparición de una coloración rosada en la pulpa que más adelante se vuelve marrón (Fig. 11C). Esta alteración en su fase avanzada da lugar a la descomposición interna, lo que conlleva que la pulpa se ablande y tenga un aspecto acuoso. Normalmente se asocia con fruta cosechada demasiado madura y también a un metabolismo fermentativo durante la conservación (Deuchande et al., 2017; Franck et al., 2007).

- Deshidratación: la piel de la pera se vuelve menos elástica, con menor brillo y se arruga; sobre todo se empieza a observar en la zona del pedúnculo. Esta alteración se debe a una baja humedad relativa durante el almacenamiento y a una refrigeración lenta (Calvo y Candan, 2013).

- Congelación: presencia de tejido translúcido debido a la formación de hielo en el tejido intracelular. Todas las frutas tienen una temperatura crítica por debajo de la cual se producen cambios no deseables y daños, es por esto que la temperatura de almacenamiento siempre debe ser mayor que la temperatura crítica de cada especie (Saquet, 2019). Como es obvio, la congelación aparece cuando la temperatura en la cámara es inferior a la temperatura crítica.

- Daños por CO_2 o corazón pardo: la pera es una especie muy sensible a niveles bajos de O_2 y altos de CO_2, por lo que niveles elevados de CO_2 pueden dar lugar a la aparición de manchas oscuras en general secas que se inician en el corazón y a la aparición de zonas de pulpa colapsadas o cavernas que pueden ser centradas en el corazón o tener una difusión radial (Fig. 11D) (Balenciaga et al., 2017; Franck et al., 2003).

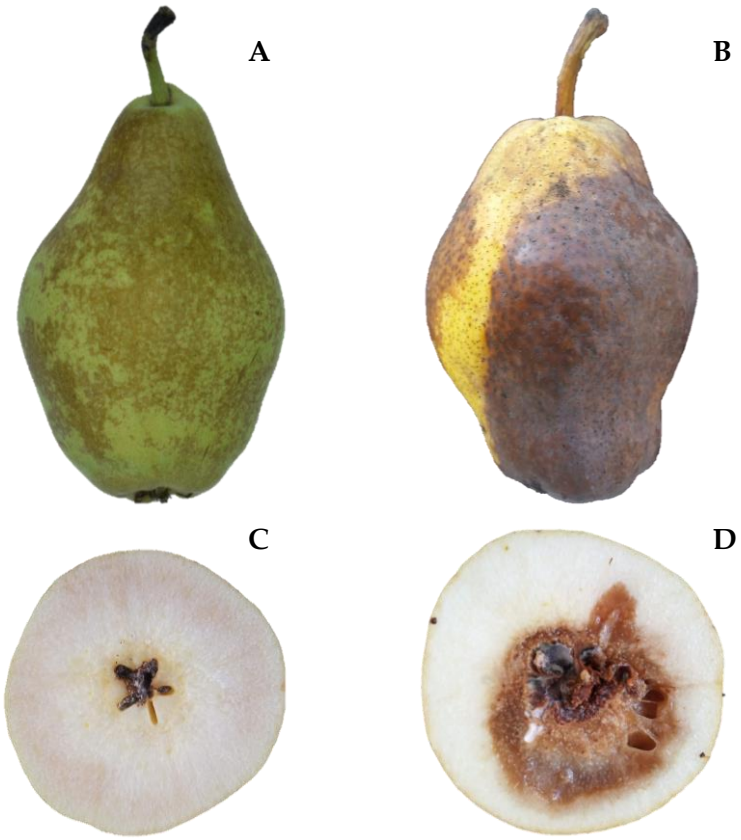

Figura 11. Ejemplos de algunas alteraciones fisiológicas: escaldado superficial en "Blanquilla" (A), escaldado de senescencia en "Williams" (B), pardeamiento interno en "Flor d'Hivern" (C) y corazón pardo en "Flor d'Hivern" (D).

2. El escaldado superficial

2.1. Descripción del desorden fisiológico

El escaldado superficial es una de las principales fisiopatías de pera y manzana que se asocia a daños por frío. Se caracteriza por la aparición de manchas marrones en la piel de la fruta que, en general, no llegan a afectar a la pulpa (Fig. 12). A pesar de que en la fruta dañada la pulpa está intacta y mantiene la textura y el sabor, esta alteración provoca grandes pérdidas económicas debido a que el consumidor no la compra. A principios del siglo XX se publicó el primer estudio donde se hablaba de unas manchas de tono marrón que aparecían en algunas variedades de manzana tras un periodo largo de almacenamiento en frío (Powell y Fulton, 1903). Es una alteración

que aparece durante la vida útil del fruto y raramente se observa a la salida de cámara (Lurie y Watkins, 2012).

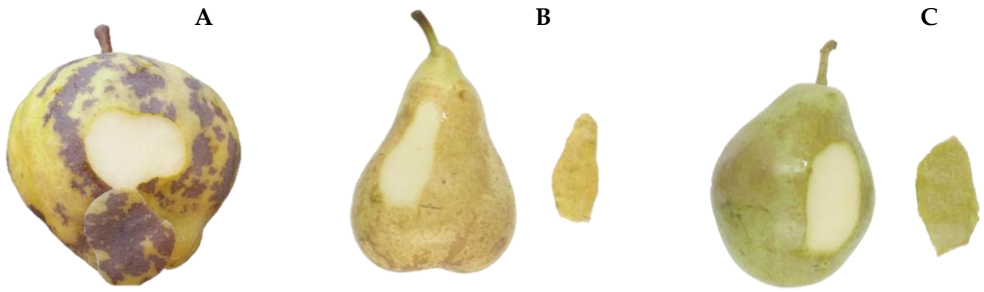

Figura 12. Variedades "Flor d'Hivern" (A), "Conference" (B) y "Blanquilla" afectadas por escaldado superficial donde se puede observar la ausencia de alteración en la pulpa.

Hay que destacar que el modelo de manzana es el más utilizado para el estudio del escaldado superficial y existe relativamente poca información acerca del escaldado superficial en pera; sin embargo, las investigaciones en pera parecen indicar que esta alteración no sigue los mismos patrones que los descritos para manzana (Larrigaudière et al., 2016).

2.2. Principales factores que determinan la aparición del escaldado superficial

- Variedad. Tanto en pera como en manzana hay variedades que son altamente susceptibles al escaldado. Dentro de las variedades de pera muy sensibles al escaldado nos encontramos con "Blanquilla", "Flor d'Hivern", "Abate Fetel" y "Alejandrina", entre otras. Las variedades de pera totalmente resistentes son difíciles de encontrar, pero sí hay algunas variedades, como "Conference", cuyo porcentaje de frutos afectados no es muy alto.

- Madurez. Una de las primeras diferencias entre el modelo de pera y manzana se encuentra en este factor. En manzana está bien establecido que la madurez en cosecha determina de forma importante la incidencia de escaldado superficial, siendo más sensibles la fruta cosechada en un estadio inmaduro (Emongor et al., 1994); sin embargo, en algunas variedades de pera se ha visto lo contrario, observando mayor incidencia de escaldado superficial a mayor madurez en cosecha (Calvo et al., 2015).

Con esto se recalca la importancia de seleccionar una fecha de cosecha óptima, no sólo para mantener la fruta más tiempo en cámara sino también para evitar la incidencia de alteraciones fisiológicas.

- Clima. En manzana, las altas temperaturas y un clima seco se asocian con una mayor predisposición al escaldo superficial, sobre todo durante las semanas anteriores a la cosecha (Emongor et al., 1994). Sin embargo, este factor no está tan detallado en pera por lo que no se sabe muy bien a qué nivel puede llegar a afectar. Teniendo en cuenta esto, es muy probable que la susceptibilidad también dependa de las condiciones edafoclimáticas donde es cultivada la fruta.

3. Teorías de la aparición de escaldado superficial

El escaldado sigue siendo objeto de estudio, sobre todo en peras, ya que todavía no se han descrito sus bases fisiológicas y bioquímicas, sino que se suelen hacer extrapolaciones del modelo establecido en manzana. Aunque se desconoce de forma detallada el porqué de este desorden en pera, existen un par de teorías que tienen bastante fuerza: la teoría del α-farneseno y la teoría del proceso oxidativo.

3.1. Teoría del α-farneseno

La teoría del α-farneseno es la más estudiada y afianzada para el modelo de manzana (Anet, 1972; Whitaker et al., 2000). Esta teoría establece que el escaldado superficial se debe a la acumulación de α-farneseno durante el almacenamiento en frío y que luego este producto se oxida a la salida de cámara, durante la vida útil, formando productos de oxidación que podrían estar relacionados con la aparición de la coloración marrón a la piel (Fig.13).

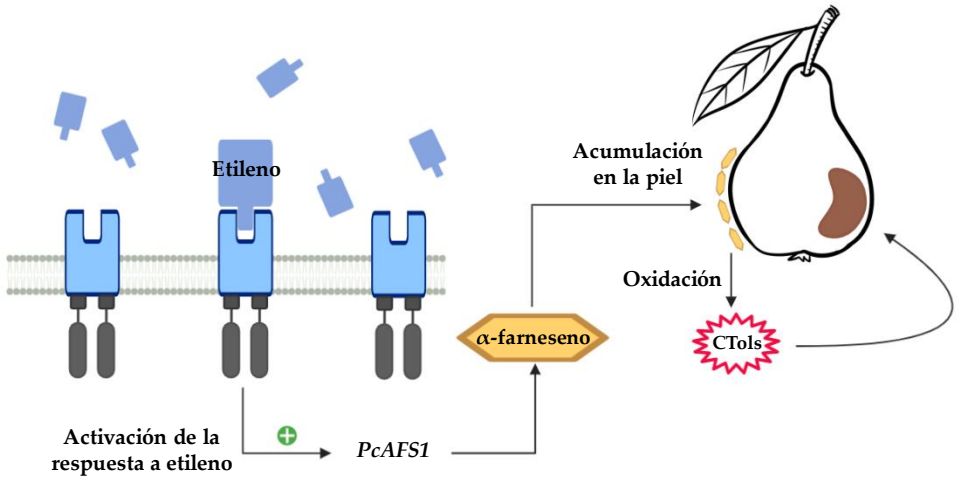

Figura 13. Esquema de la teoría del α-farneseno y la implicación del etileno en el desarrollo del escaldado superficial. Figura creada con BioRender.com.

3.1.1. Biosíntesis del α-farneseno

El α-farneseno es un sesquiterpeno (isoprenoide de 15 átomos de carbono), por lo que su ruta de síntesis pertenece a la de los terpenoides. El precursor de todos los isoprenoides es el mevalonato, el cual se forma a partir del 3-hidroxil-3-metilglutaril-Co enzima A (HMG-CoA) gracias a la acción de la enzima hidroximetilgrlutaril-coA reductasa (HMGR), y el HMG-CoA se forma mediante la condensación de 3 unidades de acetil-CoA. Después, el mevalonato da lugar al isopentenil difosfato (IPP), el cual se fusionará para formar los diferentes terpenos. Una vez formado, el IPP se condensa con geranil difosfato (GPP) para dar lugar al farnesil difosfato (FPP) gracias a la farnesil difosfato sintasa (FDS). Por último, el FPP se transforma en α-farneseno gracias a la acción de la α-farneseno sintasa (AFS) (Fig. 14). El α-farneseno finalmente puede sufrir diferentes procesos de auto-oxidación, dando lugar a los trienoles conjugados (CTols).

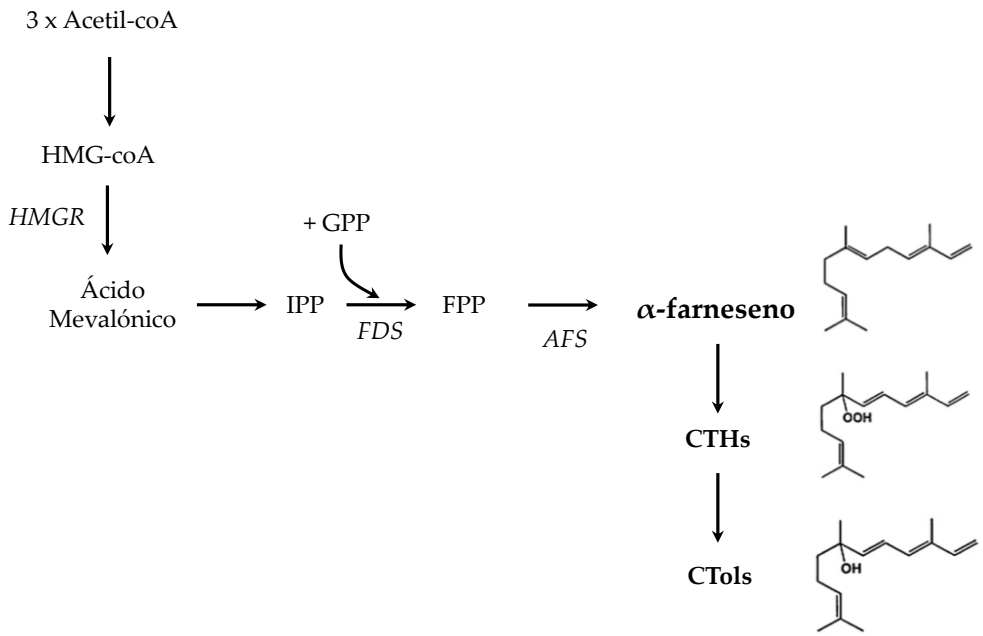

Figura 14. Ruta de síntesis del α-farneseno. Figura adaptada de Lurie y Watkins (2012).

3.1.2. Productos de oxidación

Como ya hemos comentado, el α-farneseno se puede oxidar y dar lugar a trienos conjugados (CTs). En primer lugar se forman unos compuestos intermediarios hidroperóxidos (CTHs), que son oxidados en trienoles conjugados (CTols), los cuales ya son moléculas más estables; sin embargo, el porqué de esta oxidación es aún desconocido (Lurie y Watkins, 2012). Estos trienos conjugados absorben en el rango de luz ultravioleta, observándose picos de máxima absorbancia a 258, 269 y 281 nm (Whitaker, 2004). Hay estudios que destacan la importancia del trieno conjugado con pico de absorbancia a 281 nm (CT_{281}), ya que se ha observado una correlación positiva entre este compuesto y la incidencia del escaldado superficial (Anet y Coggiola, 1974; Moggia et al., 2010). Sin embargo, otros autores demostraron que lo que realmente está relacionado con la incidencia del escaldado es el ratio CT_{258}/CT_{281} (Du y Bramlage, 1993; Zhang y Shu, 2003). Un ratio alto indica poca susceptibilidad al escaldado, aunque los valores del CT_{281} sean altos (Lurie y Watkins, 2012). Whitaker et al. (1998, 2001) mostraron que el CT_{258} es un éster del ácido *p*-cumárico, el cual puede actuar como antioxidante, de ahí su importancia en el desarrollo del escaldado. Respecto al CT_{269}, Du y Bramlage (1993) observaron que el ratio CT_{269}/CT_{281} era similar al ratio CT_{258}/CT_{281}, pero las diferencias no eran tan obvias cuando se comparaban variedades

con diferentes susceptibilidades al escaldado; por lo tanto, ambos autores propusieron que los CT_{258} y CT_{281} no son compuestos individuales sino que representan a un grupo de compuestos mientras que el CT_{269} está influenciado por ambos grupos, de ahí la similitud de los ratios. Por otro lado, Busatto et al. (2018, 2014) propusieron que, como consecuencia de un estrés oxidativo, el α-farneseno es oxidado a CTs y éstos podrían actuar como moléculas de señalización para activar un mecanismo de defensa basado en la acumulación de polifenoles y, en concreto de ácido clorogénico. La aparición de la coloración marrón sería el resultado de la oxidación de este ácido por la polifenoloxidasa (PPO).

3.1.3. Relación con el etileno

La reducción del contenido en α-farneseno y CTs mediante la aplicación de compuestos inhibidores del etileno, como el 1-metilciclopropeno (1-MCP), demuestra que el etileno está implicado en el metabolismo del α-farneseno (Isidoro y Almeida, 2006; Moggia et al., 2010).

Diferentes estudios demostraron que, durante la maduración, la concentración de α-farneseno aumentaba de manera paralela a la de etileno (Du y Bramlage, 1994a; Watkins et al., 1993). Durante años se consideró la HMGR como enzima clave en la producción de α-farneseno y se pensaba que era la que estaba inducida por el etileno (Rupasinghe et al., 2001). Sin embargo, y gracias a diferentes estudios realizados en manzana, se ha visto que es el gen de la enzima α-farneseno sintasa (AFS), encargada de transformar el farnesil difosfato en α-farneseno, el que está altamente regulado por el etileno y que los niveles de α-farneseno y CTols siguen el mismo patrón que la acumulación de los transcritos de este gen (Lurie et al., 2005; Pechous et al., 2005; Tsantili et al., 2007). Resultados similares han sido obtenidos en la variedad de pera "Beurré d'Anjou", pudiéndose extrapolar lo sucedido en manzana a pera (Gapper et al., 2006). Por otro lado, Pesis et al. (2009) demostraron que manzanas transgénicas con la enzima ACC oxidasa inhibida mostraban altas concentraciones de α-farneseno. Estos resultados, que en principio pueden parecer contradictorios, indican que incluso el etileno residual producido por estas manzanas es suficiente para inducir la expresión génica del gen de la AFS.

3.2. Teoría del proceso oxidativo

A pesar de que la teoría del α-farneseno es la más estudiada, algunos estudios han demostrado que el escaldado superficial es más bien el resultado de la acción directa de un estrés oxidativo que de la acumulación de α-farneseno y los CTols en sí. Esta hipótesis tendría bastante sentido ya que la teoría del α-farneseno se basa en un proceso de oxidación. En un estudio realizado con manzanas por Rao et al. (1998) no se vio relación entre el ratio CT_{258}/CT_{281} y la susceptibilidad al escaldado superficial, pero sí se observó que las variedades resistentes, a pesar de mostrar niveles altos de CTs, no acumulaban H_2O_2. Este hecho podría dar a entender que la mayor o menor susceptibilidad al escaldado podría estar relacionada con la capacidad del fruto de metabolizar las especies activas del oxígeno. Dos años más tarde, Whitaker et al. (2000) obtuvieron resultados similares, demostrando que los productos de oxidación del α-farneseno no son los responsables directos del escaldado superficial, pero que sí pueden agravarlo. Esto podría deberse a la función que tienen los CTs como moléculas de señalización, promoviendo la acumulación de polifenoles, los cuales producen la coloración marrón al ser oxidados (Busatto et al., 2018, 2014).

Las especies reactivas del oxígeno (ROS, de sus siglas en inglés) se producen durante la senescencia y también como respuesta a factores de estrés como las bajas temperaturas (Apel y Hirt, 2004). A pesar de que estas especies pueden causar daños celulares severos tanto a nivel de proteínas, ADN y lípidos, algunos estudios demuestran que también pueden actuar como moléculas de señalización (Apel y Hirt, 2004; Gechev y Hille, 2005). Por ello, resulta importante mantener un buen equilibrio entre los procesos de degradación y de generación de las ROS (Sharma et al., 2012), para así evitar un estrés oxidativo y el desarrollo de desórdenes fisiológicos en conservación. Dentro de las especies reactivas del oxígeno nos encontramos con radicales libres (superóxido, 0_2^-, y grupos hidroxilo,$0H^-$), oxígeno singlete ($^1O_2$) y peróxido de hidrógeno (H_2O_2) (Gupta et al., 2018) y los compuestos antioxidantes responsables de metabolizar estas especies pueden ser enzimáticos o no.

3.2.1. Enzimas antioxidantes

- Superóxido dismutasa (SOD). Esta enzima se encarga de la transformación de radicales superóxido en oxígeno molecular y peróxido de hidrógeno (del Río et al., 2018). Las bajas temperaturas inducen gradualmente la actividad de esta enzima en especies como el arroz (Oidaira et al., 2000), produciéndose una acumulación de H_2O_2

que si no es rápidamente metabolizado puede causar daños, de ahí la importancia de las enzimas degradadoras del H_2O_2. De hecho, en un estudio realizado con manzanas se vio que variedades resistentes al escaldado superficial tenían un menor contenido en H_2O_2 que aquellas variedades susceptibles, aun teniendo actividades SOD prácticamente idénticas. Se concluyó por lo tanto que lo que marcaba la diferencia eran las enzimas encargadas de metabolizar el H_2O_2 y no directamente la SOD (Rao et al., 1998). Debido a esto, la SOD no se relaciona directamente con la incidencia del escaldado superficial, tal y como se ha demostrado con diferentes variedades de manzana (Du y Bramlage, 1994b).

- Catalasa (CAT). Es la enzima responsable de atrapar dos moléculas de H_2O_2 y transformarlas en agua y oxígeno y, al igual que la SOD, también aumenta su actividad con temperaturas bajas (Leung, 2018). Algunos estudios en manzana han correlacionado negativamente la actividad de esta enzima con la incidencia del escaldado superficial (Du y Bramlage, 1995; Rao et al., 1998) reforzando la idea de que niveles bajos de H_2O_2 se asocian con variedades más resistentes al escaldado superficial. En pera, algunos estudios llevados a cabo con variedades susceptibles al escaldado superficial, también han mostrado actividad catalasa más baja en fruta control respecto a los frutos tratados con 1-MCP que no desarrollaban escaldado (Gao et al., 2015; Larrigaudière et al., 2004).

- Ascorbato peroxidasa (APX). Otra forma de degradar el H_2O_2 es mediante las peroxidasas, dentro de las cuales nos podemos encontrar con diferentes tipos en función de cual sea su sustrato. APX es la encargada de reducir dos moléculas de H_2O_2 usando como donador de electrones el ascorbato y formando agua y monodehidroascorbato (MDHA) (Sharma et al., 2012). La APX es una enzima clave en el ciclo glutatión-ascorbato, el cual forma parte de los mecanismos de defensa antioxidantes de la planta y, aunque se desconoce de qué manera puede influir exactamente la APX en el desarrollo del escaldado superficial (Zubini et al., 2007), algunos estudios resaltan la importancia de las diferentes enzimas que forman parte de este ciclo en el desarrollo del escaldado en pera (Giné-Bordonaba et al., 2020; L. Wang et al., 2018). Sin embargo, en pera sí se ha visto cierta similitud con los patrones de otras enzimas como la catalasa, observándose valores más bajos en fruta control que en fruta tratada con 1-MCP (Gao et al., 2015; Larrigaudière et al., 2004).

- Guayacol peroxidasa (POX). Esta peroxidasa utiliza como donante de electrones el fenol guayacaol (donador artificial), el cual da una coloración rojiza

cuando es oxidado (Veljović Jovanović et al., 2018); por lo tanto, en esta reacción se reducen dos moléculas de H_2O_2 dando lugar a agua y a guayacol oxidado. A pesar de que en un principio, se asoció la POX como uno de los mecanismos de resistencia frente al escaldado superficial (Rao et al., 1998), Fernández-Trujillo et al. (2003) demostraron que esa hipótesis no siempre se cumple y que, además, depende de la madurez de la fruta.

- Otras enzimas involucradas en el ciclo del glutatión-ascorbato. Aunque sea de manera rápida, no queremos olvidarnos de mencionar otras enzimas de este ciclo como son la glutatión reductasa (GR), dehidroascorbato reductasa (DHAR) y monodehidroascorbato reductasa (MDHAR). El glutatión es un tripéptido (Glutamato-Cisteína-Glicina) que en su forma reducida actúa, entre otras muchas funciones, contra la formación de ROS. La GR se encarga de transformar la forma oxidada del glutatión (disulfuro de glutatión, GSSG) en su forma reducida (GSH) (Zuccarelli y Freschi, 2018). Por otro lado, la MDHAR tiene la función vital de regenerar los niveles de ascorbato (AsA), mediante la reducción del ascorbato oxidado (monodehidroascorbato, MDHA). Este ascorbato oxidado también puede descomponerse de manera espontánea en dehidroascorbato (DHA), el cual se transforma en ascorbato gracias a la acción de la DHAR, la cual consume glutatión reducido (Zuccarelli y Freschi, 2018).

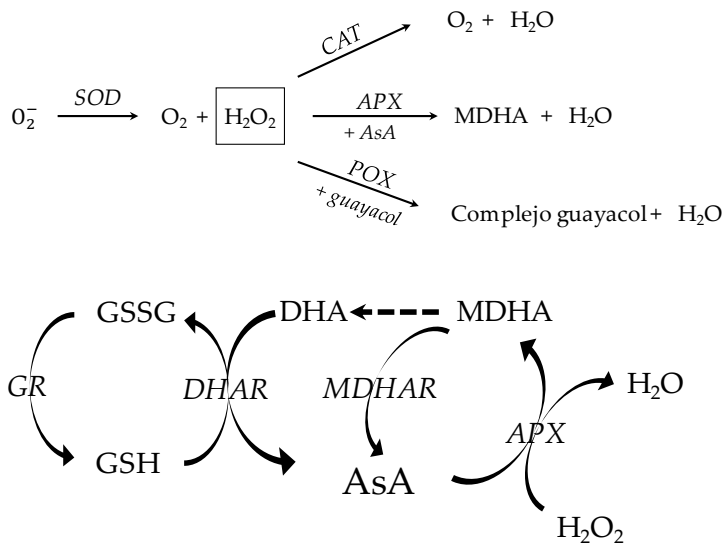

Figura 15. Esquema de las principales enzimas involucradas en la Teoría del proceso oxidativo.

3.2.2. Compuestos antioxidantes no enzimáticos

- Ascorbato (AsA). El ácido ascórbico es una molécula pequeña pero no por ello menos importante, ya que presenta muchas funciones entre las cuales podemos destacar: antioxidante en el ciclo glutatión-ascorbato, co-factor de algunas enzimas (como por ejemplo la ACC oxidasa) y también participa en procesos redox (Ishikawa et al., 2018). En nuestro caso destacaremos la función antioxidante, ya que es una molécula clave en la eliminación de especies reactivas del oxígeno (Sharma et al., 2012). Algunos estudios en pera han relacionado los bajos niveles de ácido ascórbico con una mayor incidencia de alteraciones fisiológicas (Larrigaudière et al., 2016, 2004; Veltman et al., 1999; L. Wang et al., 2018).

- Compuestos fenólicos. Son un grupo muy diverso de metabolitos secundarios que presentan cierta actividad antioxidante y se caracterizan por tener al menos un grupo fenol. Dentro de este grupo podemos destacar los flavonoides, taninos, fenilpropanoides y ligninas, entre muchos otros (Sharma et al., 2012). El metabolismo de los fenoles requiere especial atención ya que tiene un papel dual; por un lado los compuestos fenólicos pueden actuar como antioxidantes, pero durante la senescencia entran en contacto con la principal enzima de su metabolismo, la polifenoloxidasa (PPO), la cual oxida estos compuestos dando lugar a quinonas, compuestos muy reactivos que forman polímeros de coloración marrón (Franck et al., 2007). Estudios en manzana han destacado la importancia de algunos compuestos fenólicos (como el ácido clorogénico, catequina, epicatequina, etc.) debido a su participación en el desarrollo del escaldado superficial (Busatto et al., 2018, 2014). Un estudio reciente en pera reafirma esta hipótesis, recalcando la importancia del metabolismo fenólico en el desarrollo del escaldado superficial (Zhou et al., 2020).

4. Métodos de control del escaldado superficial

A continuación hablaremos sobre algunos de los métodos más utilizados para prevenir o disminuir la incidencia del escaldado superficial. En general, hay que tener en cuenta que la eficacia del tratamiento puede variar puesto que la aparición del escaldado superficial depende de la especie, el cultivar, el año de cosecha, la región del cultivo y otros muchos factores que hacen difícil encontrar un único tratamiento que sea efectivo y no tenga consecuencias negativas en la maduración del fruto. Además de estos factores, a la hora de evaluar si un tratamiento es efectivo o no, también hay que tener en cuenta el número de frutos que se están evaluando e incluso

considerar que, en la mayoría de los casos, la severidad de la alteración se analiza de manera visual y, por lo tanto, es un análisis subjetivo (Lurie y Watkins, 2012).

4.1. Control de la temperatura de almacenamiento

El almacenamiento de la pera a baja temperatura (<10 °C) tiene por objetivos: I) inducir la maduración de aquellas variedades que no son capaces de hacerlo en cosecha y II) hacer que la fruta se mantenga en buenas condiciones para su distribución. Por lo tanto, la temperatura es uno de los factores principales a tener en cuenta en el almacenamiento de la fruta.

La pera europea se caracteriza por aguantar largos periodos almacenada; de hecho, diferentes estudios han demostrado que, a una temperatura de -1 a 0 °C y una humedad relativa superior al 90 %, la pera se puede almacenar hasta 6 meses (Saquet, 2019). Temperaturas inferiores a -1 °C pueden provocar la congelación de la fruta y temperaturas superiores a 10 °C pueden acelerar la maduración de la fruta en exceso. A pesar de que la temperatura óptima de almacenamiento es entre -1 y 0 °C, algunos estudios llevados a cabo con temperaturas superiores (5 y 10 °C) permitieron reducir el tiempo necesario para inducir su capacidad de maduración (Sugar y Einhorn, 2011); por lo tanto, la temperatura de almacenamiento se puede ajustar en función al destino de la fruta (si está prevista para un consumo a corto plazo o no). Debido a que un estrés por bajas temperaturas puede provocar daño celular (Sharma et al., 2012), algunos autores proponen el llamado "acondicionamiento a bajas temperaturas". Este tratamiento consiste en almacenar la fruta durante unos días a temperaturas de 5 - 10 °C y después pasarla a 0 °C, así se han obtenido buenos resultados en pera asiática contra la aparición de las manchas marrones en la piel (Li et al., 2017; J. Wang et al., 2017). Este método también es eficaz en la prevención de los daños por frío en varias especies como mango (Zhang et al., 2017), pomelo (Chaudhary et al., 2014), granada (Kashash et al., 2016) y melocotón (Zhou et al., 2000; Infante et al., 2009). También se ha probado su eficacia contra el escaldado superficial en manzanas (Moggia et al., 2009) pero poca información hay acerca de su efecto sobre el escaldado superficial en pera europea.

4.2. Aplicación de productos químicos

- Antioxidantes. Durante muchos años, en Europa se utilizaron los antioxidantes difenilamina (DPA) y etoxiquina como productos antiescaldantes ya que tenían una efectividad alta en manzana y pera, respectivamente (Johnson et al.,

1980). Sin embargo, la prohibición a finales del año 2009 del uso de la DPA y 2 años más tarde del de la etoxiquina en toda la Unión Europea, hizo que todo el esfuerzo se centrase en buscar alternativas a estos productos (*EU pesticides Database*).

- Inhibidores de etileno. La aminoetoxivinilglicina (AVG) es un inhibidor de la ACC sintasa que se comercializa bajo el nombre de ReTain® (Lurie, 2008). Se suele aplicar en precosecha y su uso está muy extendido en manzanas, sobre todo para prevenir la caída del fruto (Lurie, 2008). En algunos estudios se ha demostrado que, además de inhibir la síntesis de etileno, el AVG también inhibe la acumulación de α-farneseno (Ju y Bramlage, 2000; Ju y Curry, 2000a), inhibiendo o retrasando la aparición de escaldado superficial tal y como se ha visto en manzana (Fadhil y Al-Bamarny, 2010). En pera se usa para prevenir la caída del fruto y también para mejorar la calidad del mismo (Butar y Çetinbaş, 2017; Çetinbaş, 2018), aunque también se ha visto su efecto en la disminución de la incidencia de desórdenes internos (Lee et al., 2014).

Sin embargo, el producto estrella para inhibir la producción de etileno es el 1-metilciclopropeno (1-MCP), comercializado para frutos como SmartFresh™ o Harvista™, dependiendo del tipo de aplicación. Este compuesto se descubrió a finales del siglo XX, gracias a los estudios de Serek et al. (1994) y Sisler et al. (1997, 1996). El 1-MCP es una molécula sencilla (C_4H_6) que tiene una estructura muy similar a la del etileno y se une a sus receptores de manera irreversible con una afinidad hasta 10 veces mayor (Handa et al., 2012). El 1-MCP está aprobado para una gran variedad de especies (manzana, pera, albaricoque, aguacate, ciruela, melocotón, etc.) pero nosotros nos centraremos en los resultados obtenidos en pera. El 1-MCP tiene un gran efecto en la prevención del escaldado superficial (Almeida et al., 2016; Calvo et al., 2018; Zhi y Dong, 2018); pero además: inhibe o retrasa la pérdida de firmeza (Lum et al., 2017; Saquet y Almeida, 2017a), inhibe la producción de etileno y la tasa respiratoria (Argenta et al., 2003; Chiriboga et al., 2012; Villalobos Acuña et al., 2011) y aumenta la actividad de enzimas antioxidantes (Chiriboga et al., 2013a; Larrigaudière et al., 2004). Por otro lado se ha visto que, si se aplica en condiciones óptimas, no altera el contenido en sólidos solubles (Calvo y Sozzi, 2004) ni el aroma y sabor en general, tal y como se demostró en un estudio donde los consumidores preferían la fruta (pera de la variedad "Packham's Triumph") tratada con 1-MCP y almacenada en frío frente a la que había sido almacenada en atmósfera controlada sin tratar (Moya-León et al., 2006).

La principal problemática del 1-MCP en pera es que algunas variedades pierden su capacidad para madurar durante su vida útil después de la conservación, lo que comúnmente se conoce como el comportamiento *evergreen*. Este comportamiento se observa principalmente en la variedad "Conference", aunque también se ha visto en otras variedades como "Blanquilla". Un tratamiento con calor después del periodo de almacenamiento en frío revierte el efecto en la variedad "Blanquilla", pero no en "Conference" (Chiriboga et al., 2010). En "Conference", el tratamiento más efectivo para revertir el *evergreen* y devolverle a la fruta su capacidad para madurar es la aplicación simultánea de 1-MCP y etileno (Chiriboga et al., 2013b, 2011). Para evitar este comportamiento *evergreen* es muy importante seguir de forma estricta las recomendaciones de Agrofresh. Por ejemplo, para el caso de "Blanquilla" la fruta debe tener un valor de firmeza mínimo de 5,5 kg y el plazo entre la cosecha y la aplicación debe ser como máximo 7 días. En el caso de "Conference", la fruta debe ser cosechada a unos valores de firmeza de 5,5-6,5 kg y también el plazo máximo entre cosecha y aplicación de 7 días, aunque se recomienda evitar tratar las peras nada más son cosechadas (E. Dupille, R&D Manager in Agrofresh Inc. Spain, Portugal and Moroco; comunicación personal).

- Otros productos. Algunos estudios apuntan hacia la aplicación de inhibidores del α-farneseno para retardar o prevenir la aparición de escaldado superficial. La lovastatina afecta a la síntesis del α-farneseno mediante la inhibición de la enzima HMG-coA reductasa (HMGR) y ha dado resultados en la disminución de la incidencia del escaldado superficial, sin afectar a la capacidad de maduración de pera y manzana (Giné-Bordonaba et al., 2020; Ju y Curry, 2000b, 2000c).

4.3. Atmósferas controladas

Otro método utilizado para mantener la calidad de la fruta, extender el periodo de almacenamiento y evitar la aparición de daños por frío, es disminuir la concentración de oxígeno y aumentar la de dióxido de carbono para reducir el metabolismo de las células (Nath y Panwar, 2018). Las concentraciones óptimas de O_2 y CO_2 varían en función de la variedad a tratar y también de la zona de cultivo. Las concentraciones de oxígeno suelen ser entre 2 – 3 %, mientras que la concentración de CO_2 es más variable (0,8 – 5 %), dependiendo de la variedad y del tiempo de conservación. Diferentes autores han demostrado que, en comparación con la atmósfera normal (21 % O_2 y trazas de CO_2), la atmósfera controlada tiene mejores resultados en diferentes variedades de pera respecto a la calidad y a la incidencia de

alteraciones fisiológicas (Almeida et al., 2016; Blaszczyk, 2010; Larrigaudière et al., 2001).

Sin embargo, la pera es una especie muy sensible a las altas concentraciones de CO_2 por lo que uno de los principales inconvenientes de esta técnica es la aparición de desórdenes internos (Saquet, 2019), el desarrollo de compuestos relacionados con un metabolismo anaeróbico (como pueden ser el etanol y el acetaldehído) y también el desarrollo de aromas desagradables (Nath y Panwar, 2018).

- Atmósfera controlada dinámica (DCA)

El concepto de atmósfera controlada ha ido evolucionando dando lugar en la actualidad a la atmósfera controlada dinámica (DCA, de sus siglas en inglés), la cual consiste en ir reduciendo la concentración de O_2 hasta el mínimo que pueda soportar el fruto sin que suponga un daño o se produzca un metabolismo fermentativo (Prange et al., 2013). Esta concentración de oxígeno se va controlando y ajustando durante todo el periodo de conservación y para ello existen diferentes técnicas: monitorizar la emisión de fluorescencia de la clorofila, monitorizar la producción de etanol o monitorizar el cociente respiratorio del fruto (Saquet, 2019). Diferentes ensayos han demostrado la efectividad de esta técnica para reducir el escaldado superficial sin afectar la calidad del fruto (Candan y Calvo, 2016; Mditshwa et al., 2017; Rizzolo et al., 2015), aunque es probable que aparezcan otros desórdenes debido a los niveles bajos de O_2 (Candan y Calvo, 2016; Mattheis y Rudell, 2011).

- Atmósfera ULO (*Ultra Low Oxygen*)

Como su propio nombre indica, la atmósfera ULO se caracteriza por mantener concentraciones muy bajas de oxígeno (Saquet, 2019), generalmente inferiores al 2 %, sin superar el límite de oxígeno tolerable por la fruta. A pesar de que esta técnica se usa principalmente en manzana (Both et al., 2016; Poirier et al., 2020), algunos estudios sí muestran su efectividad frente a ciertas alteraciones fisiológicas como el escaldado superficial (Larrigaudière et al., 2019; Saquet et al., 2017; Saquet y Almeida, 2017b). En el estudio de Larrigaudière et al. (2019) se probó la técnica xULO (*extreme* ULO), donde la fruta se almacenó en una atmósfera con 0,7 % de O_2 y 0,5 % de CO_2, y se obtuvieron muy buenos resultados frente al escaldado superficial en pera "Blanquilla". La principal ventaja de esta técnica, en comparación con el 1-MCP, es que inhibe la producción de etileno de manera parcial, permitiendo así el

ablandamiento de la pulpa y el desarrollo de otros procesos de maduración que determinan la calidad del fruto en su vida útil.

- Atmósfera ILOS (*Initial Low Oxygen Stress*)

La atmósfera ILOS se caracteriza por aplicar concentraciones extremadamente bajas de oxígeno, incluso más bajas que las utilizadas en atmósferas ULO, durante unos días (aproximadamente dos semanas) para luego aumentarlas, aunque trabajando siempre a concentraciones muy bajas. También es muy común hacer esta aplicación de manera intermitente durante las primeras semanas de almacenamiento (Prange et al., 2013; Rizzolo et al., 2015). Una presión parcial de oxígeno inferior o igual a 0,5 kPa durante 2 semanas puede inhibir la aparición de escaldado superficial en manzanas (Wang y Dilley, 2000) y pera (Calvo et al., 2002). Lo complicado de esta técnica es aplicar un estrés de bajo oxígeno que no cause daño a la pera y luego mantener la conservación en una atmósfera adecuada, que generalmente suele ser ULO.

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OBJETIVOS

El objetivo principal de la presente tesis es el **estudio de las bases fisiológicas, bioquímicas y moleculares de la maduración en pera europea y su relación con el desarrollo del escaldado superficial**. Además, un objetivo paralelo es profundizar en la fisiología de este desorden ya que, hasta el momento, la mayoría de estudios sobre el escaldado superficial están realizados en manzana.

A continuación se detallan los objetivos específicos de esta tesis:

1. Definir cómo las hormonas endógenas y sus interacciones (*cross-talk*) durante el desarrollo en campo definen las características cualitativas y el potencial de maduración de diferentes cultivares de pera (Capítulo 1).

2. Estudiar desde un punto de vista fisiológico, bioquímico y organoléptico, el patrón de maduración en árbol y poscosecha de distintas variedades de pera (peras de verano e invierno; Capítulos 2 y 3).

3. Intentar identificar un marcador bioquímico o molecular en cosecha capaz de predecir la susceptibilidad al escaldado superficial (Capítulo 4).

4. Redefinir el rol específico que juegan el etileno y el α-farneseno en el desarrollo del escaldado superficial para ver cómo estas vías metabólicas se integran en el modelo explicativo de este desorden en pera (Capítulo 5).

METODOLOGÍA

Con el fin de conseguir los objetivos establecidos, esta tesis se ha dividido en 5 capítulos. Los tres primeros capítulos tratan sobre los objetivos 1 y 2, relacionados con la fase precosecha y con la maduración de los frutos, mientras que los capítulos 4 y 5 se centran en el desarrollo del escaldado superficial. A continuación se muestra un resumen de la metodología, puesto que en cada capítulo se habla en detalle de ella.

<u>Objetivo 1</u>. Estudio del perfil hormonal y de los principales cambios fisiológicos y bioquímicos durante el desarrollo del fruto en el árbol de dos variedades que presentan diferentes necesidades de frío para madurar correctamente (Capítulo 1).

Para este primer capítulo, se hizo un seguimiento de la floración en árboles de "Blanquilla" y "Conference" en una finca comercial de Alcoletge (Lleida). Se consideró plena floración cuando al menos el 50 % de las flores estaban abiertas y, a partir de ahí, se cosecharon frutos libres de defectos a diferentes tiempos del desarrollo, referenciados con su número de días después de plena floración (DDPF). Con los frutos recién cosechados se llevaron a cabo los análisis siguientes (Figura 1 y Tabla 1).

- Producción de etileno
- Seguimiento de la evolución del peso y diámetro de los frutos
- Parámetros de calidad. A partir de 70 DDPF se realizaron medidas del valor I_{AD}, firmeza, sólidos solubles, acidez e índice de almidón
- Un lote de frutos se almacenó en frío normal durante 15 días para evaluar la producción de etileno tras dicho periodo
- De manera paralela, en cada muestreo, se congeló muestra para los diferentes análisis bioquímicos:
    - Metabolismo del etileno. Análisis de las enzimas ACS y ACO y del metabolito ACC
    - Perfil hormonal. Evolución del contenido de las hormonas ABA, IAA, GA_1, SA y JA (realizado en la Universitat de Barcelona)
    - Contenido en azúcares (fructosa, glucosa y sacarosa) y ácido málico

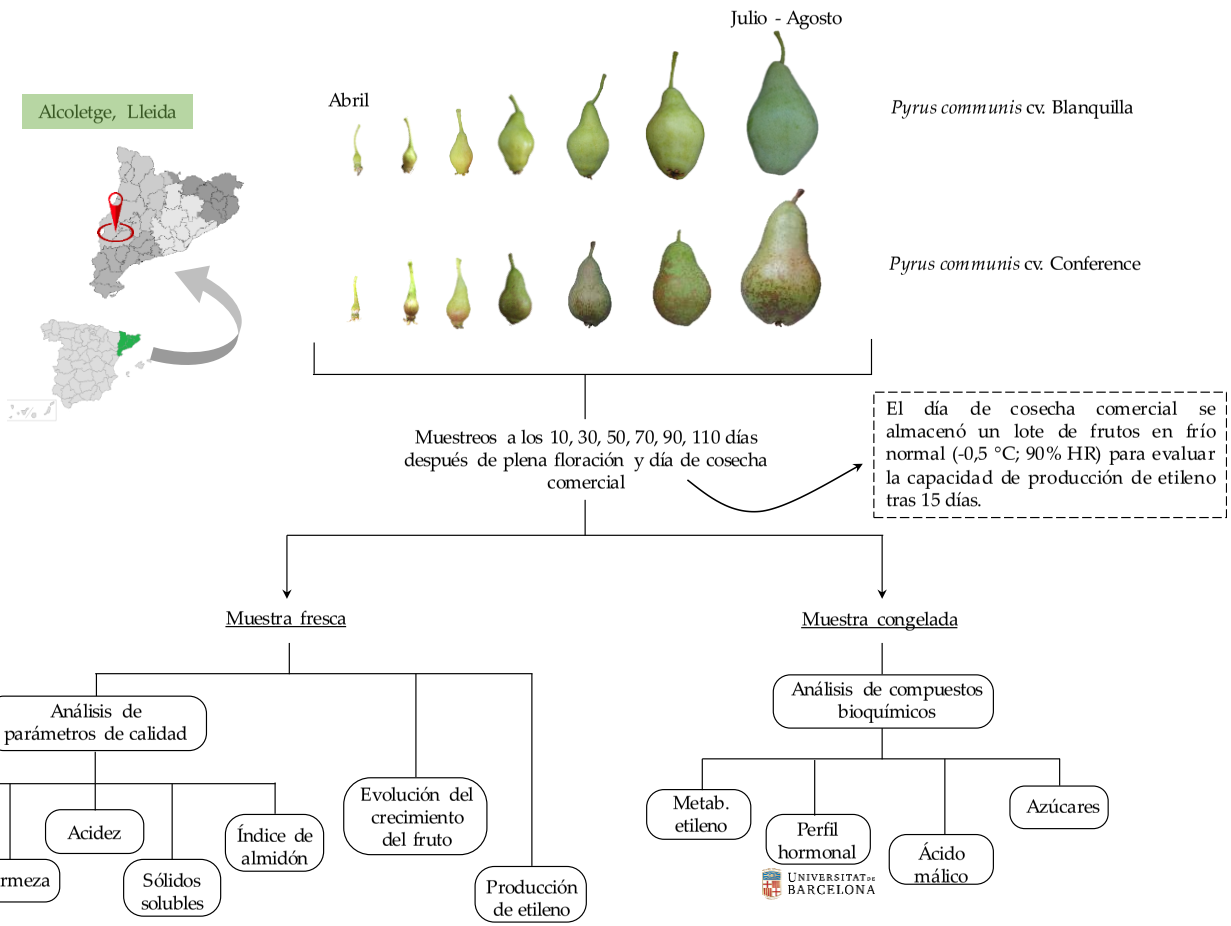

Figura 1. Estudio de la evolución de diferentes parámetros durante el desarrollo del fruto en el árbol.

<u>Objetivo 2</u>. Comparación del patrón de maduración en tres variedades de pera con diferentes comportamientos poscosecha (Capítulos 2 y 3).

En el 2º y 3er capítulo de la tesis se decidió evaluar cómo maduraban las variedades "Blanquilla" (pera típica de verano), "Conference" (pera de verano que requiere un periodo corto de almacenamiento en frío) y "Flor d'Hivern" (pera típica de invierno) en el árbol y en cámara a 20 °C durante las semanas posteriores a la cosecha comercial. En el Capítulo 2 se hace referencia al estudio realizado en la variedad "Blanquilla" y después, teniendo en cuenta los resultados obtenidos, en el Capítulo 3 se decidió hacer un estudio similar utilizando las variedades "Conference" y "Flor d'Hivern". Para ello, la fruta se dividió en dos lotes: un lote fue cosechado el día de cosecha comercial y almacenado en cámara a 20 °C, y otro lote se dejó madurando en el árbol. Se establecieron diferentes días de muestreo en los cuales se llevaron a cabo diferentes análisis (Figura 2 y Tabla 1):

- Producción de etileno
- Contenido en peróxidos en piel (solo en "Conference" y "Flor d'Hivern").
- Parámetros de calidad (I_{AD}, Firmeza, sólidos solubles, acidez e índice de almidón)
- De manera paralela se congeló muestra para los diferentes análisis bioquímicos:
    - Metabolismo del etileno. Análisis de las enzimas ACS y ACO y del metabolito ACC (este último sólo en "Conference" y "Flor d'Hivern")
    - Malondialdehído (indicador de peroxidación lipídica)
    - Capacidad antioxidante (solo en "Conference" y "Flor d'Hivern")
    - Contenido en azúcares (fructosa, glucosa y sacarosa) y ácido málico
    - Perfil volátil (sólo en "Blanquilla")

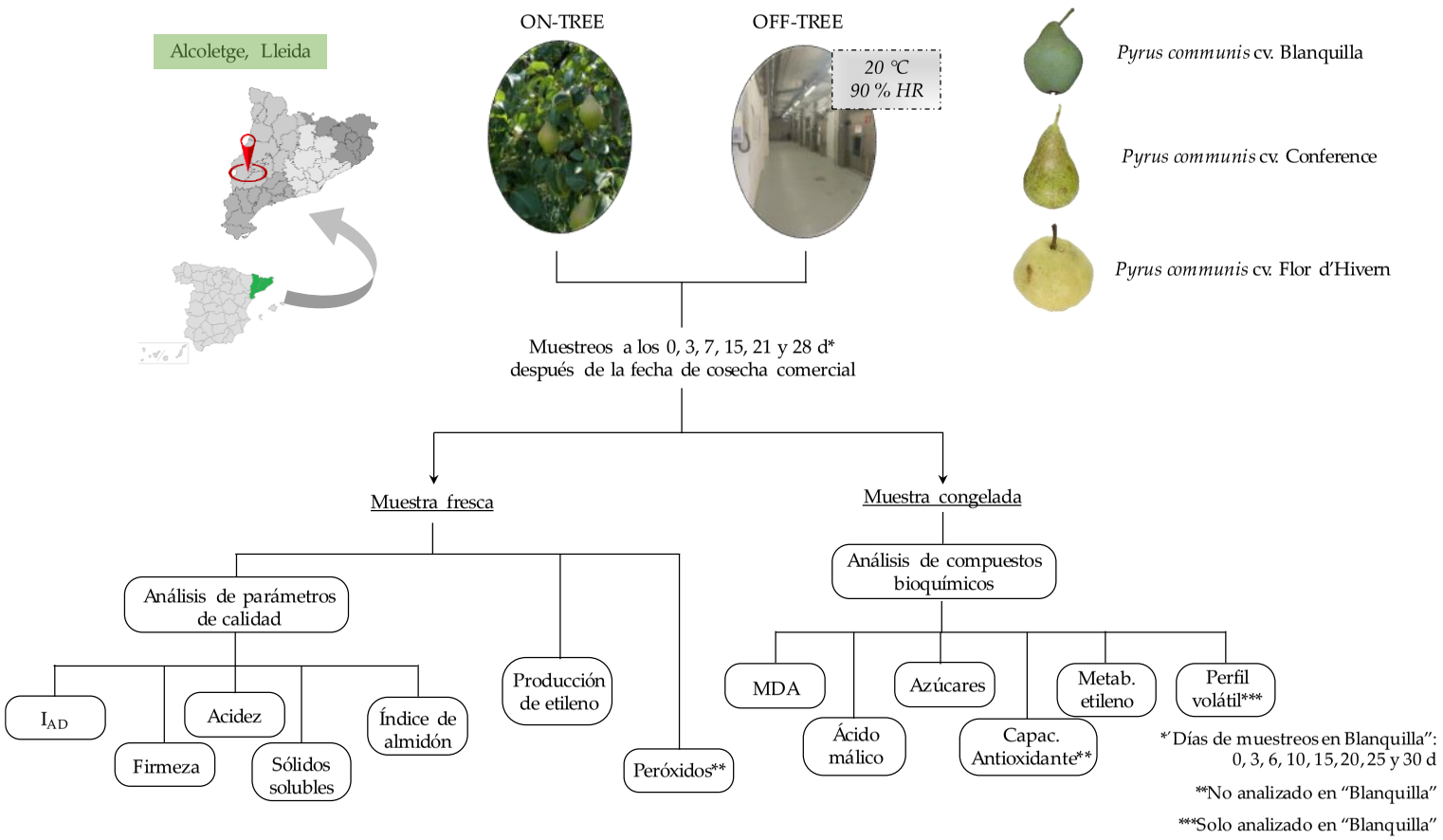

Figura 2. Comparación del patrón de maduración en árbol y fuera de él de las tres variedades estudiadas

<u>Objetivo 3</u>. Búsqueda de un marcador en cosecha capaz de predecir la susceptibilidad del fruto al escaldado superficial (Capítulo 4).

Con el objetivo de buscar algún parámetro que pudiese servir como marcador del escaldado superficial en cosecha, se establecieron cuatro fechas de cosechas cercanas a la comercial para llevar a cabo diferentes análisis. En cada una de las fechas de recolección, los frutos se dividieron en dos lotes: unos fueron directos al laboratorio para realizar los diferentes análisis y otro lote se almacenó en frío normal (-0,5 °C y 90 % HR) para evaluación de producción de etileno e incidencia del escaldado superficial tras 1 y 4 meses, respectivamente (Figura 3 y Tabla 1).

En cada fecha de cosecha se analizó:

- Producción de etileno
- Contenido en α-farneseno y CTols
- Parámetros de calidad (firmeza, sólidos solubles, acidez e índice de almidón)
- De manera paralela se congeló muestra para los diferentes análisis bioquímicos:
    - Metabolismo del etileno. Análisis de las enzimas ACS y ACO y de los metabolitos ACC y MACC
    - Metabolismo antioxidante (capacidad antioxidante, contenido en ácido ascórbico y actividad enzimática de LOX, CAT, POX y PPO)
    - Contenido en azúcares (fructosa y sorbitol)
    - Análisis de expresión génica (*PcACS1*, *PcACO1*, *PcAFS1* y *PcSDH*)
- Con la fruta que estaba almacenada en frío normal:
    - Un lote de frutos se sacó tras 30 días de almacenamiento para evaluar la cinética de producción de etileno
    - Se evaluó la incidencia del escaldado superficial tras 4 meses de almacenamiento (Figura 4)

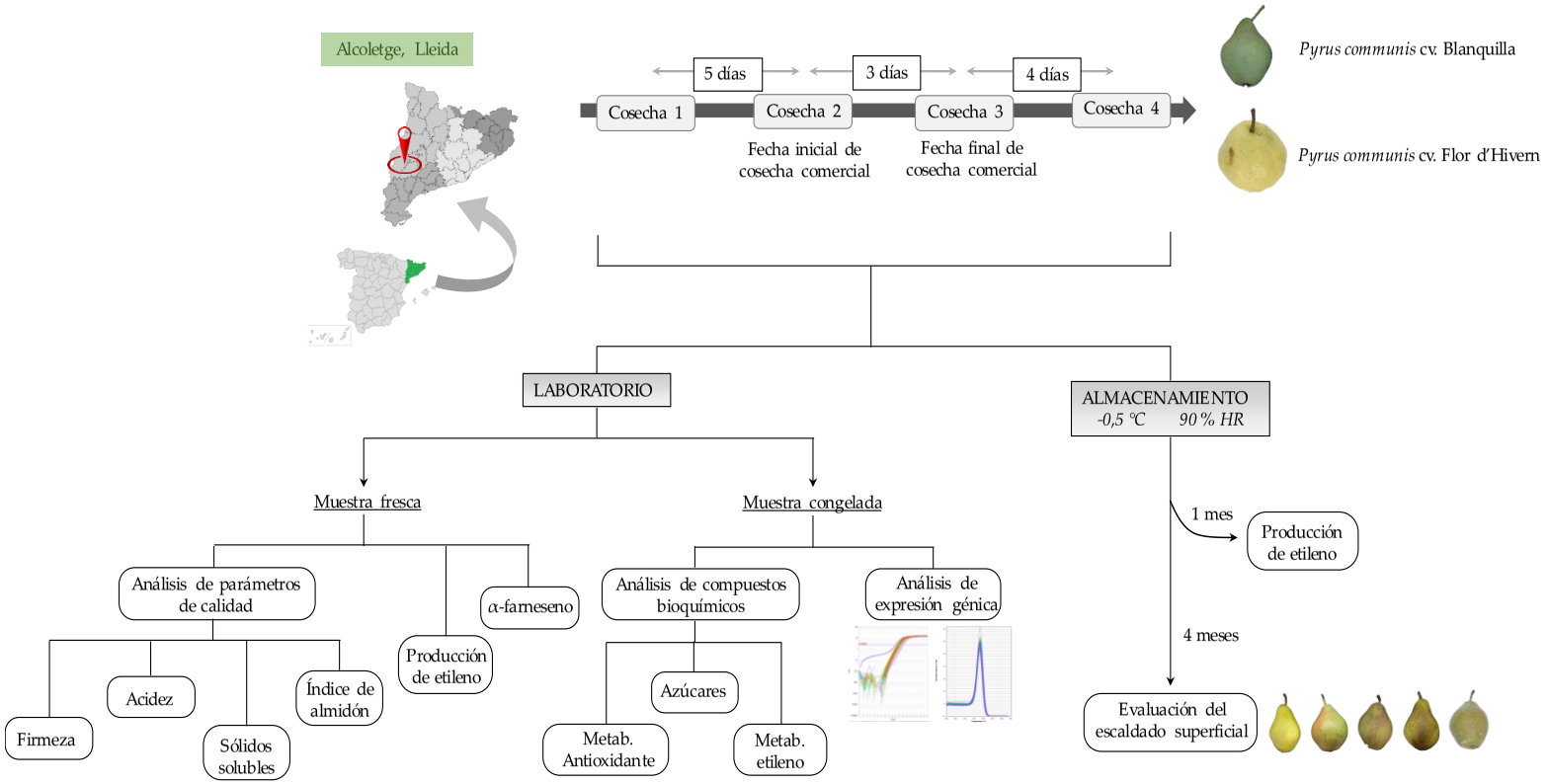

Figura 3. Búsqueda de un marcador en cosecha capaz de predecir la susceptibilidad al escaldado superficial.

Figura 4. Escala utilizada para la evaluación visual del escaldado superficial, donde: S1 = <10 % de la superficie del fruto está afectada, S2 = 10-25 %, S3 = 25-50% y S4 = >50 % de la superficie está escaldada.

<u>Objetivo 4</u>. Estudiar la relación del etileno y el α-farneseno en el desarrollo del escaldado superficial en tres variedades con comportamientos poscosecha totalmente distintos (Capítulo 5).

El objetivo de este capítulo era estudiar los cambios del metabolismo del etileno y el α-farneseno durante el almacenamiento en frío para definir sus roles específicos en el desarrollo del escaldado superficial. Para ello, se trabajó con 3 lotes de frutos: uno se dejó sin tratar (control), otro lote se trató con 0,3 μL L^{-1} de 1-MCP y el tercero se trató con lovastatina a una concentración de 1,25 mmol L^{-1} (Figura 5). Después de aplicar los tratamientos, la fruta se almacenó en frío normal (-0,5 °C y 90 % HR) y se establecieron diferentes puntos de muestreo para llevar a cabo análisis siguientes (Figura 6 y Tabla 1).

- Parámetros de calidad en cosecha (firmeza, sólidos solubles, acidez e índice de almidón)
- Producción de etileno
- Análisis de α-farneseno y CTols
- Metabolismo del etileno. Análisis de las actividades enzimáticas de ACO y ACS y del contenido en ACC
- Análisis de expresión génica (*PcACS1*, *PcACO1*, *PcHMGR*, *PcAFS1*, *PcEIN2*, *PcERF1* y *PcETR1*)
- Evaluación de la incidencia del escaldado superficial en cada uno de los lotes tras 6 meses de almacenamiento.

Figura 5. Aplicación de los tratamientos de 1-MCP (fotos superiores; mediante difusión en contenedor cerrado herméticamente) y lovastatina (fotos inferiores; por inmersión).

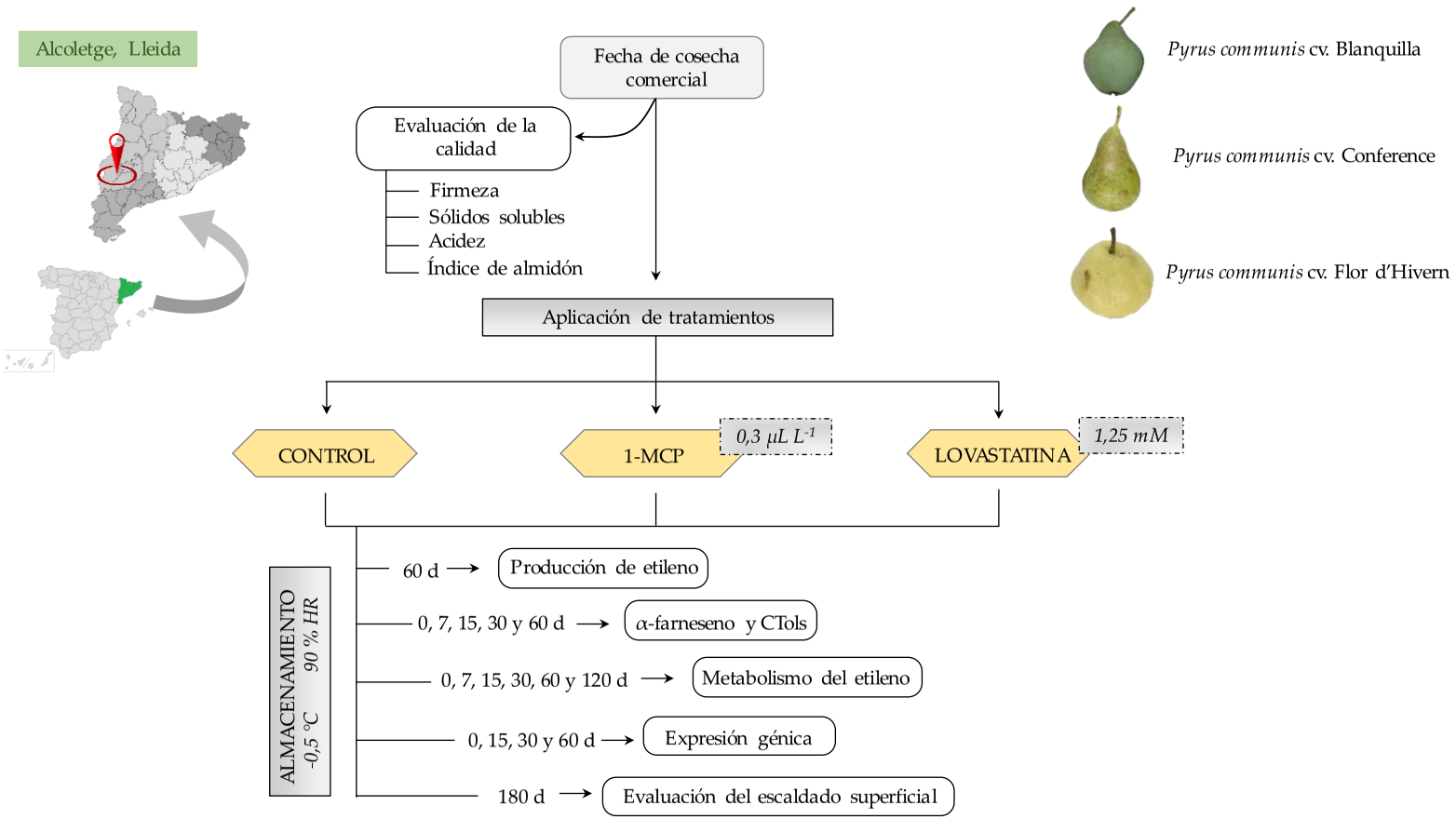

Figura 6. Metodología utilizada para investigar la relación del etileno y el α-farneseno en el desarrollo del escaldado superficial.

Tabla 1. Resumen de las técnicas analíticas utilizadas en esta tesis.

Compuesto	Anotación	Técnica análisis	Referencia	Capítulo
Etileno	Enzimas de biosíntesis de etileno	GC-FID	Giné-Bordonaba et al. (2017, 2014)	Todos
ACS		GC-FID	Chiriboga et al. (2013)	Todos
ACO		GC-FID	Chiriboga et al. (2012) y Giné-Bordonaba et al. (2017)	Todos
ACC/MACC	Precursor de la síntesis de etileno y conjugado	GC-FID	Bulens et al. (2011)	1, 3, 4 y 5/4
Azúcares y ácidos	Calidad sensorial	Espectrofotometría basada en reacción enzimática	Giné-Bordonaba et al. (2017)	1, 2, 3 y 4
α-farneseno y CTols		Espectrofotometría directa	Larrigaudière et al. (2019)	4 y 5
Perfil hormonal		UHPLC-ESI-MS/MS	Müller y Munné-Bosch (2011)	1
Perfil volátil	Calidad sensorial	SPME-GC-FID	Qin et al. (2012)	2
Malondialdehído	Procesos oxidativos y metabolismo antioxidante	Espectrofotometría basada en reacción enzimática	Martínez-Solano et al. (2005)	2 y 3
Peróxido de hidrógeno		Espectrofotometría basada en reacción enzimática	Giné-Bordonaba et al. (2017)	3
Capac. antioxidante		Espectrofotometría basada en reacción enzimática	Giné-Bordonaba and Terry (2016)	3 y 4
Ácido ascórbico		HPLC	Rassam y Laing (2005)	4
LOX		Espectrofotometría de cinética enzimática	Larrigaudière et al. (2001)	4
CAT		Espectrofotometría de reacción enzimática	Giné-Bordonaba et al. (2017)	4
POX		Espectrofotometría de cinética enzimática	Giné-Bordonaba et al. (2017)	4
PPO		Espectrofotometría de cinética enzimática	Giné-Bordonaba et al. (2017)	4
Análisis de expresión génica		RT-qPCR	Baró-Montel et al. (2019), Busatto et al. (2019), Giné-Bordonaba et al. (2020) y Muller et al. (2002)	4 y 5

GC = Gas Cromatography; FID = Flame Ionization Detector; UHPLC = Ultra High Performance Liquid Cromatography; ESI = Electrospray Ionization; MS = Mass Cromatography; SPME = Solid Phase Micro Extraction; RTqPCR = Real Time quantitative Polymerase Chain Reaction

RESULTADOS

CAPÍTULO 1

Interplay between hormones and assimilates during pear development and ripening and its relationship with the fruit postharvest behaviour

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Plant Science (2020), https://doi.org/10.1016/j.plantsci.2019.110339

Abstract

The ability of European pears (*Pyrus communis* L.) to ripen immediately after harvest is cultivar-dependent and relies on a range of physiological and biochemical events occurring during fruit growth and development that remain largely unknown. To gain further knowledge on these events, changes in the content of sugars, acids, major hormones and ethylene precursors or related enzymes were studied in two pear varieties ('Blanquilla' and 'Conference') with known differences in their postharvest ripening behaviour. In both cultivars, low contents of abscisic acid (ABA) seemed to be a prerequisite to initiate on-tree fruit ripening including sugar accumulation and softening. In 'Blanquilla' pears, the enhanced potential to produce ethylene and thereby to ripen upon harvest was associated to a late increase in ABA content paralleled by an accumulation of indole 3-acetic acid (IAA). In turn, the inability of 'Conference' fruit to produce ethylene upon harvest appeared to be related to a coordinated action of gibberellins (more specifically GA_1), salicylic acid (SA) and jasmonic acid (JA), which remained at high concentrations during the latest phases of fruit growth. Collectively, our results highlight that a complex hormonal cross-talk during the development and on-tree ripening of pear fruit may finally determine the ability of the fruit to ripen upon harvest.

Keywords: ACC, Chilling requirements, Fruit growth, Hormonal cross-talk, Sugar accumulation.

1. Introduction

Pome fruit growth followed a single-sigmoid curve [1] that on a physiological basis could be divided in four phases: ovary development, cell division, rapid growth due to cell expansion and finally ripening [2]. Some authors, though, combine the first two phases into a single one, characterised by a limited increase in fruit weight [1]. The first step along fruit development, generally referred as 'fruit set', occurs just after pollination and may be defined as the passage from flower to fruit. Cell division begins soon after blooming and is maintained for few weeks depending on the fruit and cultivar. For instance, some apple varieties complete the cell division phase in three weeks while late pear cultivars may need at least six weeks [1]. After cell division, fruit increase their weight by 100-fold or more during the so-called cell expansion period [3]. Ripening, as the last phase during on-tree fruit development, is characterised by changes in colour, texture, aroma and nutritional quality leading to the final fruit appearance and flavour.

It is well known that changes at the hormonal level are responsible, to some extent, for the transition between the above-mentioned growth phases. It has been reported, for instance, that auxins and gibberellins (GAs) play their major role at early stages of development controlling cell division and cell expansion [4]. Later on during fruit growth, auxins seem to play a primary role in the control or initiation of fruit ripening [5], yet controversial results have been found depending on the fruit species suggesting that complex interaction among different hormones are involved in the initiation of fruit ripening [6]. For instance, an inverse relationship between auxin contents and ripening capacity was observed in achenes-free strawberries treated with exogenous auxins [7], but also in the tomato ripening inhibitor (*rin*) mutant [6] and *rin*-like apple mutant [8]. Similarly, GAs synthesized during the growth phase of tomato fruit contribute to a reduced expression of genes encoding the enzymes of ethylene synthesis and also to reduced abscisic acid (ABA) synthesis [4]. ABA is one of the most important hormone in non-climacteric fruit which is involved in colour changes in grapes [9] and anthocyanins accumulation in cherries [10]. Although ethylene remained at low levels in grapes over the entire development, ABA levels gradually increased and reached their highest levels at the beginning of ripening [11]. In contrast, information regarding the involvement of ABA in the ripening process of climacteric fruit is rather scarce yet a putative role in promoting ripening has been found in tomato fruit [11].

Jasmonic acid (JA) and its methyl ester, methyl jasmonate, are other plant ubiquitous hormones that contribute to different morphogenetic events including cell division and adequate formation of tissues [12]. Jasmonates also act as growth inhibitors in root and shoots of Arabidopsis [13] and may promote climacteric fruit ripening by increasing ethylene production [14,15], chlorophyll degradation [16], the synthesis of aroma compounds [17] and the biosynthesis of several secondary metabolites and antioxidants [18] in a range of plant species. Salicylic acid (SA) is often considered as a signalling molecule that may trigger plant defence responses [19], acting both on gene expression and on the synthesis of defence compounds such as proline and JA [20]. Its putative role in fruit growth and the cross talk with others plant growth hormones is unknown although SA was found to inhibit ethylene production in pear cell suspension culture [21] as well as in canola plants [22].

Finally, ethylene is considered the major hormone involved in the ripening of climacteric fruit and numerous studies have investigated ethylene metabolism during ripening of pome fruit [23–25]. Although belonging to the climacteric class, pears are generally classified as summer or winter pears depending on the way ethylene is induced after harvest. Summer pears are able to ripen, hence to produce autocatalytic ethylene, just after harvest with a minimum or no chilling requirement. This group includes cultivars such as 'Blanquilla' that may produce considerable amounts of ethylene after harvest and even on-tree [23], but also 'Rocha' pears [26]. 'Conference' pears, on the other hand, may be considered an intermediate between summer and winter pears, needing minimum chilling requirements (about 15 d) to initiate their autocatalytic ethylene production yet depending on the fruit maturity at harvest. On the other hand, winter pears such as 'Comice' and Beurré d'Anjou' need 30 d and 150 d of cold storage, respectively, to induce ethylene production and reach their eating quality upon removal from cold storage [27,28].

Although a large amount of studies have investigated the chilling requirements of winter pears [28,29], only few studies exist on the physiological basis of this process and on the biochemical events occurring on-tree that may determine the differences in the ripening behaviour among cultivars. Even less information is actually available about the potential role that phytohormones, or the complex cross-talk among them, can play in the regulation of the pear ripening capacity. Accordingly, this study aimed to determine how the hormonal and other biochemical changes occurring during fruit growth, may explain the differential postharvest behaviour of different pear varieties.

2. Material and methods

2.1. Plant materials and experimental design

'Blanquilla' and 'Conference' pears (*Pyrus communis* L.) were harvested on a commercial orchard near Lleida (Catalonia, Spain) every 20 d from 10 d after full bloom (DAFB) until the commercial harvest date (CHD, 122 DAFB in 'Blanquilla' and 132 DAFB in 'Conference'). Biochemical and physiological measurements were performed every 20 d from 30 DAFB to the CHD whereas quality determinations were initiated at 70 DAFB.

In parallel, a batch of fruit (n=20) per variety was stored at 0 °C and 90 % RH to follow the ethylene production after 15 d of cold storage.

2.2. Quality evaluations

Flesh firmness was measured on 4 replicates of 10 fruit each with a penetrometer (TR Turoni srl., Italy) equipped with an 8 mm probe as described by Chiriboga et al.[30]. Total soluble solids (TSS; %) were measured on pear juice (blend of 10 fruit per replicate and 4 replicates) using a digital hand-held refractometer (Atago, Tokyo, Japan) whereas total acidity (TA) was measured on the same juice samples by titration using NaOH 0.1N and the results expressed as g malic L^{-1}. The index of absorbance difference ($I_{AD} = A_{670} – A_{720}$) was measured on opposite sides of the equatorial parts of the fruit with a DA-Meter (TR Turoni, Forli, Italy). The starch index was evaluated on 10 fruit samples as described by Lindo-García et al. [23]. Weight was also monitored on 6 replicates of 10 fruit each during all sampling dates. In parallel, from 30 DAFB, tissue from 5 fruit per replicate and 4 replicates (6 replicates for hormones assay) per sampling date was frozen in liquid nitrogen and kept at -80 °C until further biochemical analysis.

2.3. Ethylene production and respiration rate

Ethylene production levels (nmol kg^{-1} s^{-1}) during growth were measured as described by Giné-Bordonaba et al. [31]. Four replicates of 5 fruit each were placed in different flasks sealed with a silicon septum for sampling the gas of the headspace after 3 h incubation in an acclimatized chamber at 20 °C. Gas samples (1 mL) were taken using a syringe and injected into a gas chromatograph (GC; Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina

column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain). Fruit respiration was determined by quantifying the CO_2 concentration in the flask with an O_2/CO_2 gas analyser (CheckPoint O_2/CO_2, PBI Dansensor, Ringsted, Denmark).

Kinetics of ethylene production upon harvest and after chilling were assessed using a flow-through system according to Giné-Bordonaba et al. [32]. The ethylene production rate was determined on four replicates of two pears each placed in 1500 mL flasks continuously ventilated with humidified air at a flow rate of approximately 1.5 L h^{-1}. As previously, ethylene production was measured by taking gas samples of effluent air from respiration jars and injecting this sample into a gas chromatograph.

2.4. Sugar and organic acid content

Sugars (sucrose, glucose and fructose) and malic acid were extracted from frozen tissue as described by Giné-Bordonaba et al. [31]. The supernatants of each sample extraction were recovered and used for enzyme coupled spectrophotometric determination of glucose and fructose (hexokinase/phosphoglucose isomerase), sucrose (β-fructosidase) and malic acid (L-malate dehydrogenase) using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer's instructions.

2.5. Enzymes related to ethylene metabolism

1-aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) enzymes were extracted and analysed as described by Lindo-García et al. [23]. Enzyme activity was expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh weight basis.

1-aminocyclopropane-1-carboxylic acid (ACC) was extracted and analysed as described by Bulens et al. [33] with some modifications. Briefly, 2 g of frozen tissue were homogenized with 4 mL of a 5 % (w/v) sulfosalicylic acid solution and vortexed until a homogenous mixture was obtained. The samples were gently shaken for 30 min at 4 °C and then were centrifuged at 8,000 *g* for 10 min at 4 °C. Subsequently, the supernatant was stored at -80 °C until analysis. The extract reading was performed mixing 1.4 mL of the ACC extract with 400 μL of 10 mmol L^{-1} $HgCl_2$ and 200 μL of a solution of NaOCl saturated with NaOH (2:1 v/v). After 4 min, a 1 mL

headspace gas sample was injected into a gas chromatograph and the results expressed as nmol C_2H_4 kg^{-1} on fresh weight basis.

2.6. Hormonal profile

Phytohormones were extracted by mixing 100 mg of the fruit samples with 200 mL methanol:isopropanol:acetic acid, 50:49:1 (v/v/v) and using ultrasonication and vortexing (Branson 2510 ultrasonic cleaner, Bransonic, USA) for 30 min. Deuterium-labelled internal standards, including d5-indole-3-acetic acid, gibberellins (d2-GA_1; OlChemim Ltd. (Olomuc, Czech Republic)) and abscisic acid (d6-ABA; OlChemim) were added. After centrifugation, the pellet was re-extracted using the same procedure and the collected supernatants were merged and filtered through a 0.22 mm PTFE filter (Waters, USA) before analyses. Phytohormones were analysed by UHPLC-ESI– MS/MS. The system consisted of an Aquity UPLCTM System (Waters) quaternary pump equipped with an autosampler. An HALOTM C18 (Advanced Materials Technology Inc., USA) column (2.1 x 75 mm, 2.7 μm) was used. Solvent A was water with 0.05 % glacial acetic acid (Sigma-Aldrich, Steinheim, Germany) and solvent B was acetonitrile (Sigma-Aldrich) with 0.05 % glacial acetic acid. Flow rate was set at 0.6 mL min^{-1}. Quantification was made considering recovery rates for each sample by using the deuterium-labelled internal standards [34] and the results expressed on fresh weight basis (μg kg^{-1}) and per fruit basis (μg $fruit^{-1}$) aiming to understand the accumulation of hormones without considering the increase in fruit volume occurring during fruit growth.

2.7. Statistical analysis

All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS Institute. Comparisons between varieties at specific time points were done by Student's *t*-test at a significant level of $p \leq 0.05$. Correlations between experimental variables were made by Spearman's Rank Correlations using RStudio® and *p* value based on a two-tailed test (significant differences were $p \leq 0.05$).

3. Results

3.1. Different postharvest ripening behaviours

'Blanquilla' pear was able to produce ethylene just after harvest, reaching values of almost 0.1 nmol kg^{-1} s^{-1} after 11 d at 20 °C. In contrast, 'Conference' pears did not

produce ethylene after harvest, maintaining basal levels even after 15 d at 20 °C (Fig. 1A). After 15 d of cold storage, 'Conference' pears started to produce ethylene after 11 d at 20 °C reaching maximum levels of 0.4 nmol kg^{-1} s^{-1} after 15 d of storage at 20 °C. On the other hand, ethylene production in cold-stored and then ripened 'Blanquilla' pear was similar to that observed at harvest (Fig. 1B).

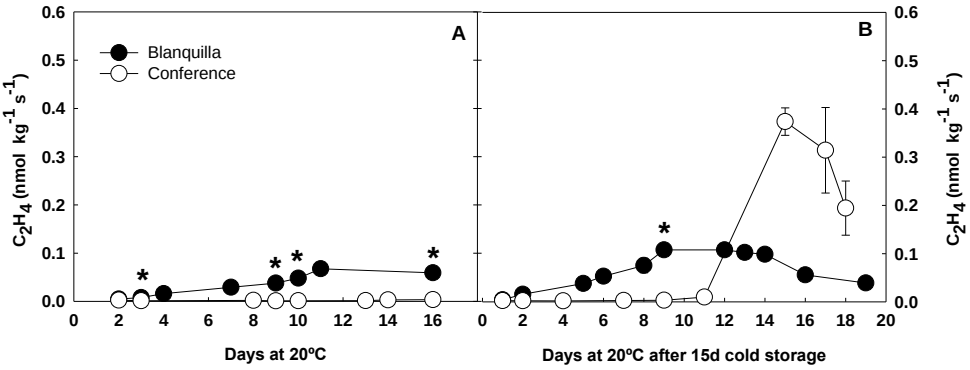

Figure 1: Changes in ethylene production at harvest (A) and after 15 d at 0 °C (B) in 'Blanquilla' (●) and 'Conference' (○) pears. Error bars represent the standard error of the means (n=4). * indicate significant differences at $p \leq 0.05$ between cultivars at a specific sampling.

3.2. Growth kinetics, morphological and quality changes

The growth pattern in weight (g) of 'Blanquilla' and 'Conference' pears along the different sampling dates is showed in Fig. 2, highlighting two clearly differentiated growth phases. The first one, occurring between 10 and 70 DAFB, showed a slow growth rate (around 0.4 and 0.7 g per day for 'Blanquilla' and 'Conference', respectively) followed by a second phase, from 70 DAFB to CHD, characterised by a faster growth rate (around 1.6 and 2.8 g per day for 'Blanquilla' and 'Conference', respectively). A marked decrease in the fruit firmness and titratable acidity along with an increase in the TSS content and the starch index (Suppl. Fig. 1) was observed for both varieties parallel to the second growth phase.

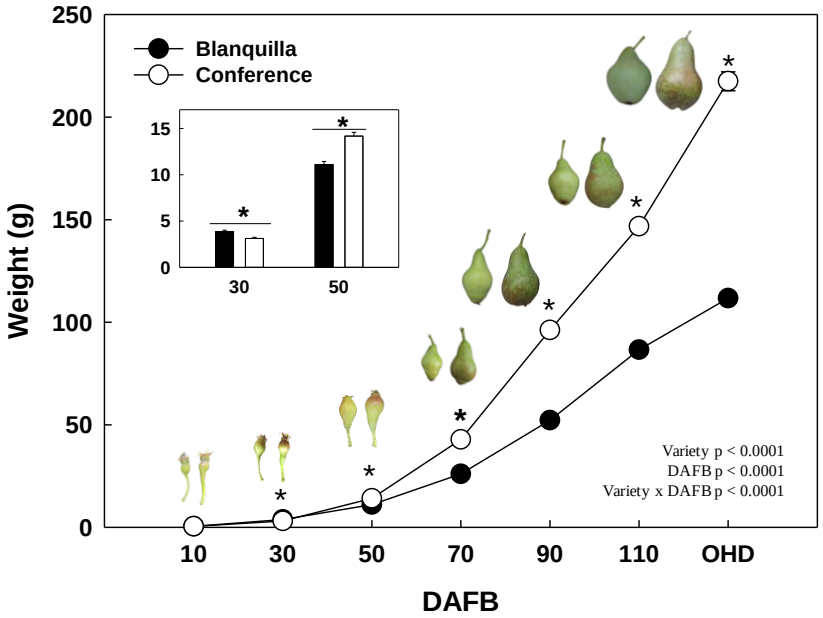

Figure 2: Evolution of fruit growth in 'Blanquilla' (●) and 'Conference' (○) pears from 10 DAFB to the time of commercial harvest (CHD). Error bars represent the standard error of the means (n=6). * indicate significant differences at $p \leq 0.05$ between cultivars at a specific sampling. Insert shows the weight of the fruit at 30 and 50 DAFB for 'Blanquilla' (black column) and 'Conference' (white column) varieties.

3.3. Sugar and organic accumulation during fruit growth

In this work, the glucose accumulation pattern was quite similar between the two varieties depicting higher glucose values in 'Conference' already from 70 DAFB (Fig. 3A) onwards. At the time of commercial harvest, glucose content was 10 and 7 g kg^{-1} for 'Conference' and 'Blanquilla' pears, respectively, being in average 4.6-fold higher than the values observed at 30 DAFB. Fructose was the predominant sugar in both varieties being maximum at 110 DAFB and slightly declining thereafter until the CHD (Fig. 3C). Sucrose content, on the other hand, remained stable until 70 DAFB and then increased by 4-fold until the CHD (Fig. 3B) parallel to the period of faster fruit growth. In turn, the minor changes in sucrose content until 70 DAFB were paralleled by the highest fruit CO_2 production and only start to rise when CO_2 production was relatively low (data not shown).

Malic acid is the predominant organic acid in pear fruit and its content both in 'Blanquilla' and 'Conference' pear was about 1.6 g kg^{-1} at 30 DAFB and then

constantly increased through fruit development (14 mg per day) reaching values of 2.5 and 3 g kg^{-1} at 110 DAFB for 'Conference' and 'Blanquilla', respectively (Fig. 3D).

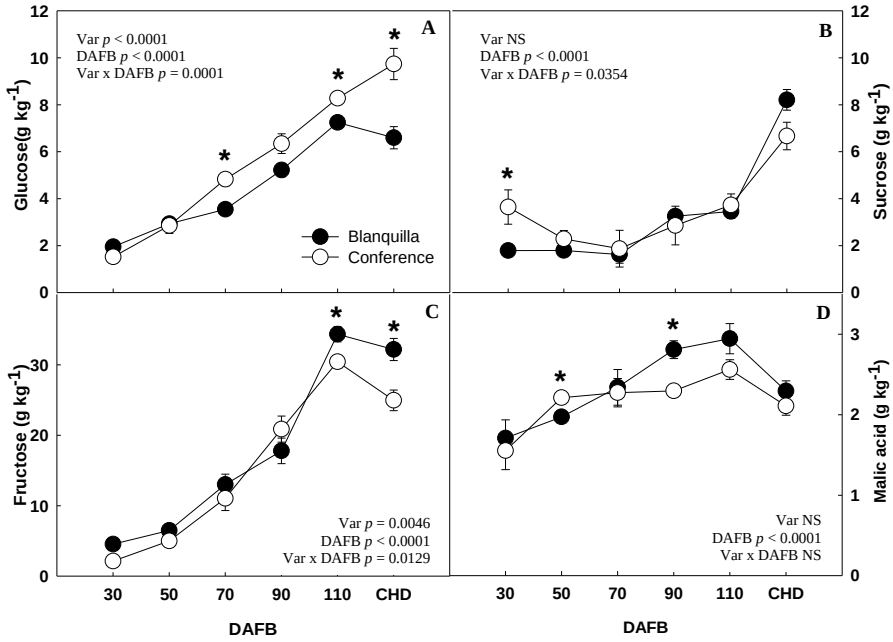

Figure 3: Changes in glucose (A), sucrose (B), fructose (C) and malic acid (D) content in 'Blanquilla' (●) and 'Conference' (○) along fruit growth and development (represented as days after full bloom; DAFB). Error bars represent the standard error of the means (n=4). * indicate significant differences at $p \leq 0.05$ between cultivars at a specific sampling.

3.4. Changes in ethylene metabolism during growth and ripening

The ethylene production pattern during fruit growth was also similar between the two varieties (Fig. 4A) observing an increase of ethylene production rate from 30 to 50 DAFB and then a decreased, reaching basal levels at 70 DAFB. Significant differences in the ethylene production rate between cultivars were observed at the CHD, where 'Blanquilla' pears were capable of producing 6.5-fold higher ethylene levels than 'Conference' pears.

Significant differences in ACC levels were found between cultivars with values of 500 nmol kg^{-1} in 'Blanquilla' at the time of commercial harvest date whereas 'Conference' exhibited 5-fold lower values (Fig. 4C). A massive increase in the ACC content of 'Blanquilla' fruit occurred from 110 DAFB to the CHD. Such differences in

the ACC levels between cultivars were not explained by an activation of ACC synthase (ACS), that remained very low throughout the full growing period for both varieties (Fig. 4B), nor by the increase in ACC oxidase activity near the commercial harvest date (Fig. 4D). Indeed, the activity of this enzyme follow a similar pattern between the two varieties.

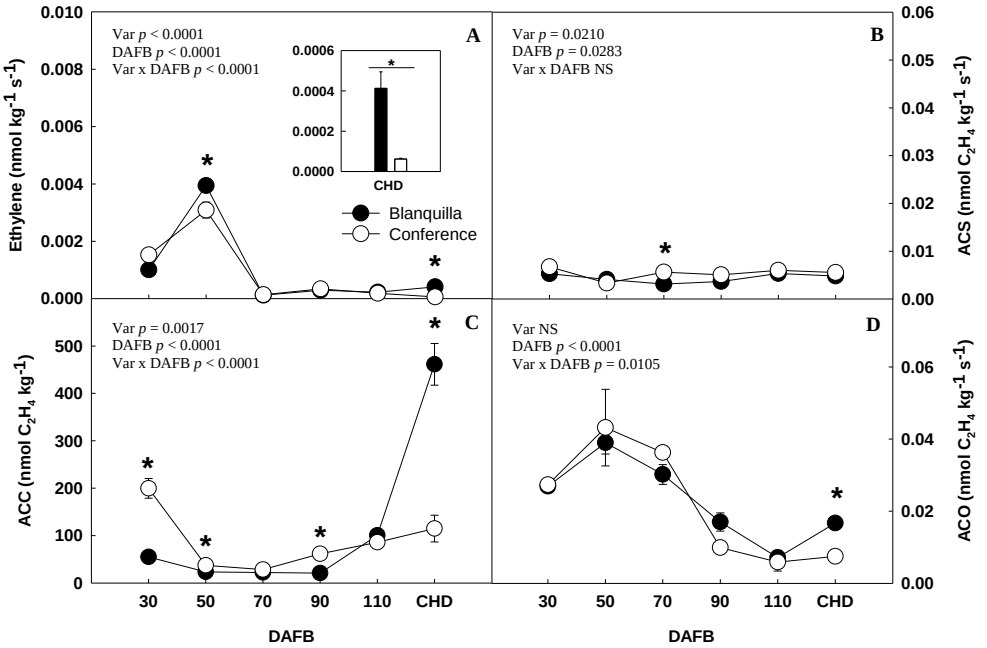

Figure 4: Changes in ethylene production (A), ACC synthase activity (B), ACC content (C) and ACC oxidase activity (D) in 'Blanquilla' (●) and 'Conference' (○) along fruit growth and development (represented as days after full bloom; DAFB). Error bars represent the standard error of the means (n=4). * indicate significant differences at $p \leq 0.05$ between cultivars at a specific sampling. Insert in Figure 4A shows the ethylene production at CHD for 'Blanquilla' (black column) and 'Conference' (white column) varieties.

3.5. Hormonal changes during pear growth and ripening

In our study, a differential accumulation pattern of ABA was observed between cultivars. For both cultivars, ABA levels were high at earlier developmental stages (*ca*. 6 mg kg^{-1} at 30 DAFB), and drastically declined thereafter until 70 DAFB (Fig. 5A). From this moment until the CHD, ABA levels continue to decline in 'Conference' pears, whereas these levels steadily increased in 'Blanquilla' pears reaching values of 1.5 mg kg^{-1}. The results expressed on a fruit basis, hence showing the net accumulation

of this hormone within the fruit, showed that ABA content in 'Blanquilla' pear increased from fruit set until the commercial harvest date whereas ABA levels in 'Conference' remained steady during most of the fruit development process (Fig. 5A, insert). It is interesting to note that in both cultivars the ABA peak during growth (observed at 30 DAFB; Fig. 5A) precede that of ethylene (50 DAFB; Fig. 4A).

The changes in the content of indole 3-acetic acid (IAA) also differed between cultivars and especially at earlier developmental stages (Fig. 5B). 'Conference' pears showed its highest value of IAA (around 40 μg kg^{-1}) at 50 DAFB whereas, at the same time, IAA levels in 'Blanquilla' were the lowest (around 20 μg kg^{-1}). During the period of maximum growth (second growth phase from 70 DAFB until the CHD), IAA levels in 'Blanquilla' pears remained stable whereas a clear decrease and significantly lower values ($p \leq 0.05$) for this hormone were observed in 'Conference' pears, yet with a transient peak at 90 DAFB. A similar behaviour was observed considering the data in fruit basis (Fig. 5B, insert).

As described for the other phytohormones, changes in the content of the most active gibberellin, GA_1, were also notably different between the two cultivars. At 30 DAFB, GA_1 levels in 'Blanquilla' pears were relatively high (12 μg kg^{-1}) but decreased later up to 5 μg kg^{-1} at 70 DAFB. A completely different accumulation pattern was found during this first growth phase in 'Conference' pears for which GA_1 levels increased from 5 μg kg^{-1} to 10 μg kg^{-1} from 30 to 70 DAFB. After 70 DAFB, GA_1 levels remained stable in 'Blanquilla' pears but slightly increased up to harvest date in 'Conference' pears (both on a concentration or fruit basis). At CHD, GA_1 levels in 'Conference' pears were 2-fold higher than in 'Blanquilla' (Fig. 5C).

JA levels remained stable (80 μg kg^{-1}) in 'Blanquilla' pears during all the growing period. However, in 'Conference' pear two peaks in JA levels were observed at 50 and 110 DAFB (Fig. 5D), and values during this second growth phase were generally higher than those observed in 'Blanquilla', and especially at 110 DAFB where significant differences ($p \leq 0.05$) were observed between varieties.. On fruit basis, a similar tendency was observed for both varieties until 70 DAFB, yet from this date onwards, 'Blanquilla' fruit also showed constantly lower JA levels than 'Conference' pear (Fig. 5D, insert). Similarly, significant differences were found in the kinetics of accumulation SA between the two cultivars during growth. 'Blanquilla' pear showed a peak of SA at 50 DAFB (around 25 μg kg^{-1}) whereas 'Conference' reached the same values at later developmental stages (90 DAFB) (Fig. 5E).

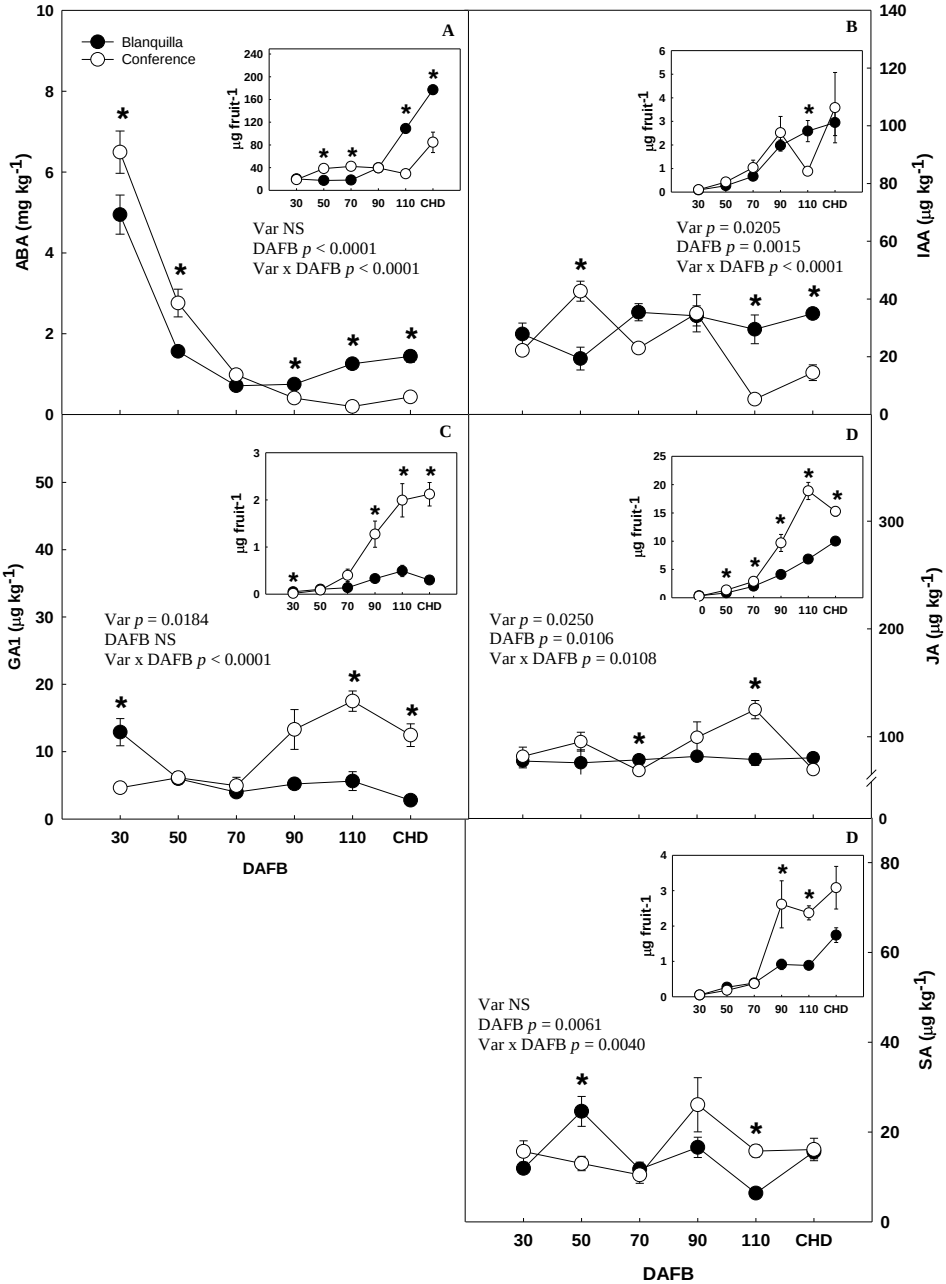

Figure 5: Endogenous concentration of abscisic acid (ABA; A), indole 3-acetic acid (IAA; B), gibberellin 1 (GA1; C), jasmonic acid (JA; D) and salicylic acid (SA; E) in 'Blanquilla' (●) and 'Conference' (○) along fruit growth and development (represented as days after full bloom; DAFB). Error bars represent the standard error of the means (n=6). Inserts describe for each graph the temporal changes on a fruit basis. * indicate significant differences at $p \leq 0.05$ between cultivars at a specific sampling.

4. Discussion

Notable differences exist in the ripening capacity and the fruit sensitivity to cold stress among different pear varieties. Our data further confirm that 'Blanquilla' pear is a typical summer pear variety able to produce ethylene just after harvest whereas 'Conference' pear needed at least 15 d of cold storage following harvest to initiate the production of ethylene. Similar results have been showed in previous studies of 'Blanquilla' pear where ethylene production increased to 0.08 nmol kg^{-1} s^{-1} after 15 d at 20 °C [23] as well as in 'Conference' [35] or other 'winter' pear varieties [36] which needed variable chilling periods to produce detectable amounts of ethylene. Even though the chilling-requirements to initiate ripening in many winter pear varieties are well documented [28,29], scarce information exists on the physiological basis of this process and on the biochemical events occurring on-tree that may determine the differences in the ripening behaviour among cultivars.

4.1. Growth rate or changes at the assimilate level do not differ between cultivars with different ripening capacity.

In spite of the notable differences in the final fruit size and postharvest behaviour both varieties showed similar growth patterns. The number of phases involved in pear growth remains controversial [1–3,24], but our results show for both varieties two clear differentiated phases, and hence agreed with a previous study in Japanese pear [37]. The first phase is likely associated to ovary development [3] and rapid cell divisions [2] whereas the second growth phase is probably related to an increase of the cell volume due to cell enlargement.

Along the changes in fruit size, sugars (glucose, fructose and sucrose) and acids tended to accumulate similarly between both varieties throughout fruit growth. A similar accumulation pattern has been described for other species such as cherry [31], loquat [38] but also apple [24]. While glucose and fructose constantly increase through fruit development, sucrose started to peak at later developmental stages coinciding with the period of low fruit respiration (data not shown). This result suggests that sucrose may be a major substrate for respiration in pear fruit, as it has been described in other plant models [39]. Higher sucrose content in 'Blanquilla' than in 'Conference' at the CHD further confirm the association between the accumulation of this compound with the initiation of the autocatalytic ethylene production on-tree [23]. In turn, the constant accumulation of malate throughout fruit development also

reinforce the results from recent studies suggesting that this compound may account little or nothing as a respiratory substrate in a range of species [40,41].

4.2. Ethylene plays a key role at different stages during pear growth

Numerous studies have investigated ethylene metabolism during ripening of pome fruit [23–25] yet most of them have focused at later fruit developmental stages. The peak in ethylene production observed at 50 DAFB is likely involved in the regulation of cell size promoting the passage from cell division to cell elongation, as suggested by Small et al. [42]. Similar patterns of ethylene production during growth have been reported in apples [24,43], observing high ethylene levels at early stages of development, followed by a notable decrease prior the commercial harvest and a final increase until the CHD. The observed differential activation of ACC levels and ACO activity clearly show that 'Blanquilla' pear was physiologically more mature at the CHD and explain why this variety does not require chilling to initiate its autocatalytic ethylene production (Fig. 1). Indeed, the higher ethylene production at harvest in 'Blanquilla' is in agreement to previous studies that demonstrate the ability of this pear variety to produce ethylene even on-tree [23]. However, in 'Conference' pears, cold storage is needed to increase the levels of ACC [44] and hence to produce significant amounts of ethylene upon chilling. Whether the differences in ACC among varieties are due to a differential capacity of each cultivar to conjugate ACC in malonyl ACC (MACC) remains to be further confirmed yet similar MACC levels are found in both varieties after cold storage (ranging from 2 to 5 nmol g^{-1}) [44]. Based on these results, and available literature [45–48], we further investigated how other phytohormones may be involved in inhibiting or triggering ACC metabolism on-tree, and hence accounting for the different ripening capacity among pear varieties.

4.3. The involvement of the hormonal cross-talk in the pear ripening behaviour

4.3.1. ABA, a key hormone involved in sugar accumulation and more indirectly in the initiation of pear ripening.

The low levels of ABA in unripe 'Blanquilla' fruit followed by the steady increase prior to commercial harvest is in agreement to the behaviour described in other climacteric fruit such as apples (cv. 'Red winesap') [49]. The high levels of ABA at 30 DAFB suggest that this hormone may own a prominent role during the first stages of pear development, and that lower levels of this hormone may be required for the transition from fruit expansion to ripening (Fig. 2). Furthermore, the peak of ABA

preceding that of ethylene agrees with the data of Zhang et al. [11] showing that a cross-talk between ABA and ethylene may be required to trigger certain stages of pear fruit growth. Although evidence suggests that ABA plays an important role as an inducer of ripening along with ethylene in the late stage of fruit development [11], very few reports are actually available on their role during the entire fruit growth. It is generally recognized that ABA levels increase from maturation to harvest in climacteric fruit, while in non-climacteric fruit such as sweet cherries, the levels of ABA increase before maturation but decrease thereafter until the time of harvest [50]. Accordingly, our data (Fig. 5A) suggest that 'Blanquilla' pears followed a typical climacteric pattern with a slight increase in ABA content at the end of the fruit growth, hence in agreement to that described in apples [50], while 'Conference' pears rather resemble a non-climacteric fruit [51]. Although both cultivars are recognized as climacteric, such differences in ABA accumulation during growth is of particular interest and may explain, at least in part, the differences in the postharvest ripening behaviour and chilling requirements of these cultivars. Based on available data, we may speculate that cold storage in 'Conference' pear can trigger the accumulation of endogenous ABA, as observed in sweet cherries [10], and thereby enable the fruit to ripen thereafter.

Besides the role of ABA in fruit ripening and its cross-talk with ethylene, several evidences exist about the involvement of ABA in sugar accumulation and starch hydrolysis, most of them related to non-climacteric fruit such citrus fruit, cherries but also in melon [6,12,49]. A recent study demonstrated that endogenous concentration of ABA increased during fruit development in sweet cherries, along with the corresponding increase in sugars such as glucose and fructose [53]. Our results confirm the relationship between sugar accumulation and ABA levels and further support recent hypothesis in which the accumulation of specific sugars was needed for the initiation of the autocatalytic ethylene production on-tree in 'Blanquilla' pears [23] through its cross-talk with ABA [54].

4.3.2. IAA influences cell division and expansion and may act as a ripening inducer

IAA is considered to play a key role in fruit development and ripening [2,48,54]. In our study, the changes in IAA varied depending on the variety. For instance, the temporal changes of IAA in the flesh of 'Conference' pears support previous studies [55], reporting that two peaks of auxin occurred during tomato fruit development.

At later fruit developmental stages, and especially during the period of maximum sugar accumulation and initiation of ethylene production, IAA levels were 2.1-fold higher in 'Blanquilla' than in 'Conference' pears confirming a putative role for auxins as essential elements in the induction of the fruit responsiveness to ethylene [56]. In other plant models, it was hypothesised that auxin-induced ethylene synthesis is actually connected to ABA and leads to growth inhibition [57]. This hypothesis may explain not only the ability of 'Blanquilla' pears to ripen immediately after harvest but also its slower size if compare to 'Conference'.

4.3.3. Gibberellin 1 (GA1) promotes fruit set but inhibits ripening

Several authors have elucidated the importance of GAs, along with auxins, during fruit growth and development by promoting fruit set [6,58]. At early stages of development, GA_1 has been related to the promotion of fruit set and growth in cherry [52], tomato [55] and Japanese pear [59]. Although auxins are known to regulate cell division and expansion, GAs have also been described to promote cell expansion in tomato fruit [60]. Along with the data regarding IAA, differences in GA_1 between both varieties may further explain why 'Conference' pears are larger than 'Blanquilla' pears at harvest. In the latest stages of fruit development, near to the CHD, GAs may act as a ripening inhibitor delaying the ripening process as it has been observed in tomato fruit [61]. Indeed, an important GA-ethylene-ABA cross-talk was found in tomatoes, where GAs at fruit set decrease the expression of ETR6, ACS and ACO genes as well as those genes related to ABA degradation [45]. In these lines, our data shows that GA_1, although undoubtedly involved in fruit growth, may also contributes to the ripening inhibition observed in 'Conference' pears by affecting the accumulation of both ethylene and ABA as reported by others [45]. Nevertheless, further studies are needed to better understand the relation of GA_1 with the accumulation of specific compound or other phytohormones as well as its putative implication in fruit ripening.

4.3.4. JA and SA may inhibit on-tree ripening and determine pear postharvest behaviour

JA and SA have been reported to play a crucial role in abiotic and biotic plant stress [15,20]. However, their involvement during fruit growth and ripening is elusive. In contrast to the results reported herein, endogenous JA concentrations decreased during apple fruit growth [17]. Thus said, in the same study, the authors

found that JA inhibited to some extent a range of ripening-related processes [17] which is consistent with our data for 'Conference' pear. In this sense, the higher net JA content in 'Conference' during the second growth phase can explain the inability of this cultivar to ripen. Kondo et al. [46] also showed that application of a JA analogue decreased the activity of ACC synthase and delayed fruit ripening in 'La France' pears. Our results are also consistent with the works of Nham et al. [62] who showed that JA may modulate the ethylene pathway through a negative regulation of EIN3 gene expression in 'Bartlett' pear.

In a similar way, SA has been described as an effective ripening inhibitor [22], affecting several ripening related processes (respiratory rate, softening, cell wall degrading enzymes, sugars accumulation, etc.) in fruit such as banana [47]. The higher levels of SA, alone or in combination with other hormones (GA_1 and JA), observed in the late stages of the 'Conference' pear growth, may explain the lower or non-detectable ethylene production produced by this cultivar at harvest, since this compound has been described to inhibit ACO enzyme activity [22,47].

5. Conclusions

The present work demonstrates that, although ethylene is considered the major hormone involved in the ripening of climacteric fruit such as pears, other hormones play a decisive role during fruit growth and development through a complex but coordinated cross-talk that may finally determine the pear ripening behaviour both on-tree and during postharvest. Accordingly, two different models are presented in Fig.6 describing the hormonal and assimilate regulation on pear fruit ripening.

In fruit capable to ripen on-tree such as 'Blanquilla', once on-tree ripening was initiated (at 70 DAFB), ABA progressively accumulates (2-fold higher at CHD that at 70 DAFB) in parallel to the fruit capacity to produce ethylene (ACC accumulation). These sustained higher levels of ABA or ethylene also likely promote the action of IAA that remained at high levels at the end of fruit growth and development for this variety.

On the other hand, the inability of 'Conference' fruit to produce ethylene, and thereby to normally ripen upon harvest, may be due to a coordinated action of GA_1, SA and JA (already known as ripening inhibitors for other species), which remained at high concentrations during the latest phases of fruit growth leading to an inhibition of the ethylene metabolism.

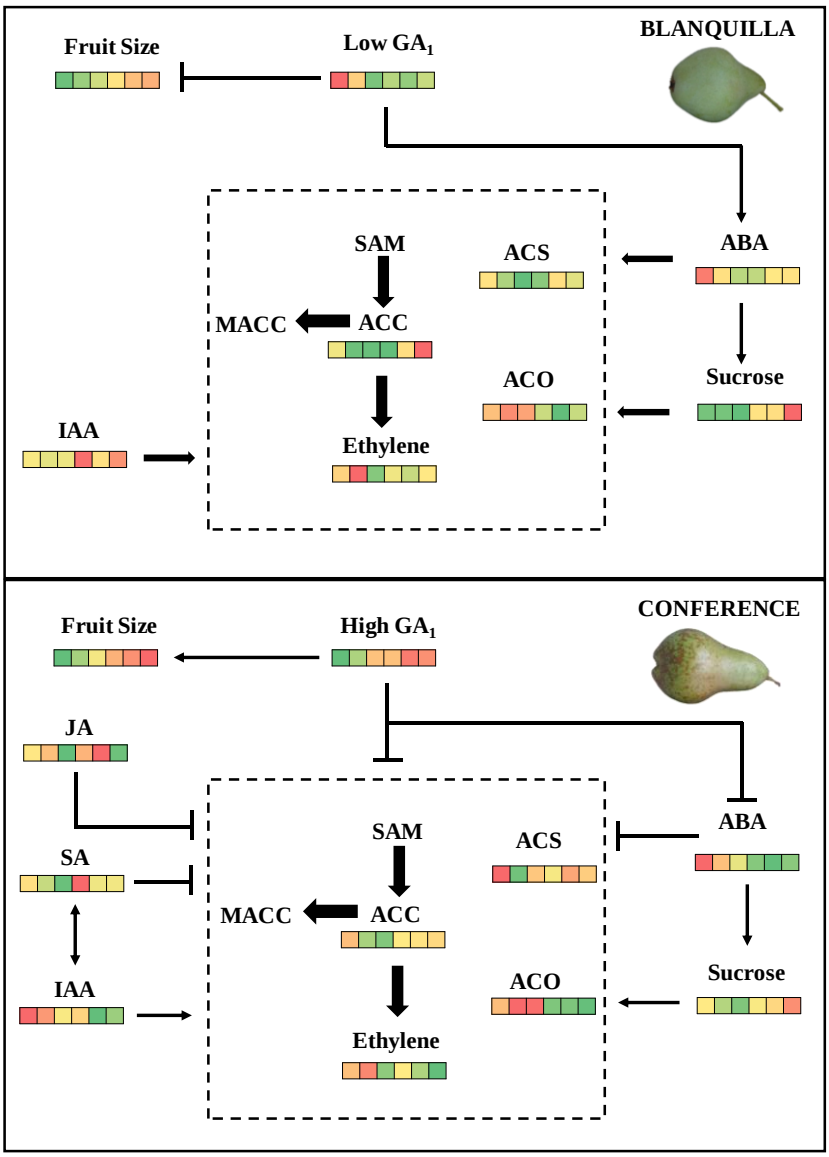

Figure 6: Proposed model for the hormonal cross-talk and its interaction with specific biochemical compounds or related physiological events in 'Blanquilla' (summer pear capable of ripening after harvest) and 'Conference' (Winter-like variety requiring a short-chilling period to initiate ripening).

6. Declaration of competing interest

All authors declare no conflict of interest. The results included in this article have not been previously published or accepted for publication and are not under consideration by any other journal.

7. Acknowledgments

This work has been supported by the CERCA Programme/Generalitat de Catalunya. SMB was supported by the Generalitat de Catalunya through the ICREA Academia award. We are grateful to Dolors Ubach and Elisabet Duaïgues for technical support and Albert Estevez for his help with the correlations matrix.

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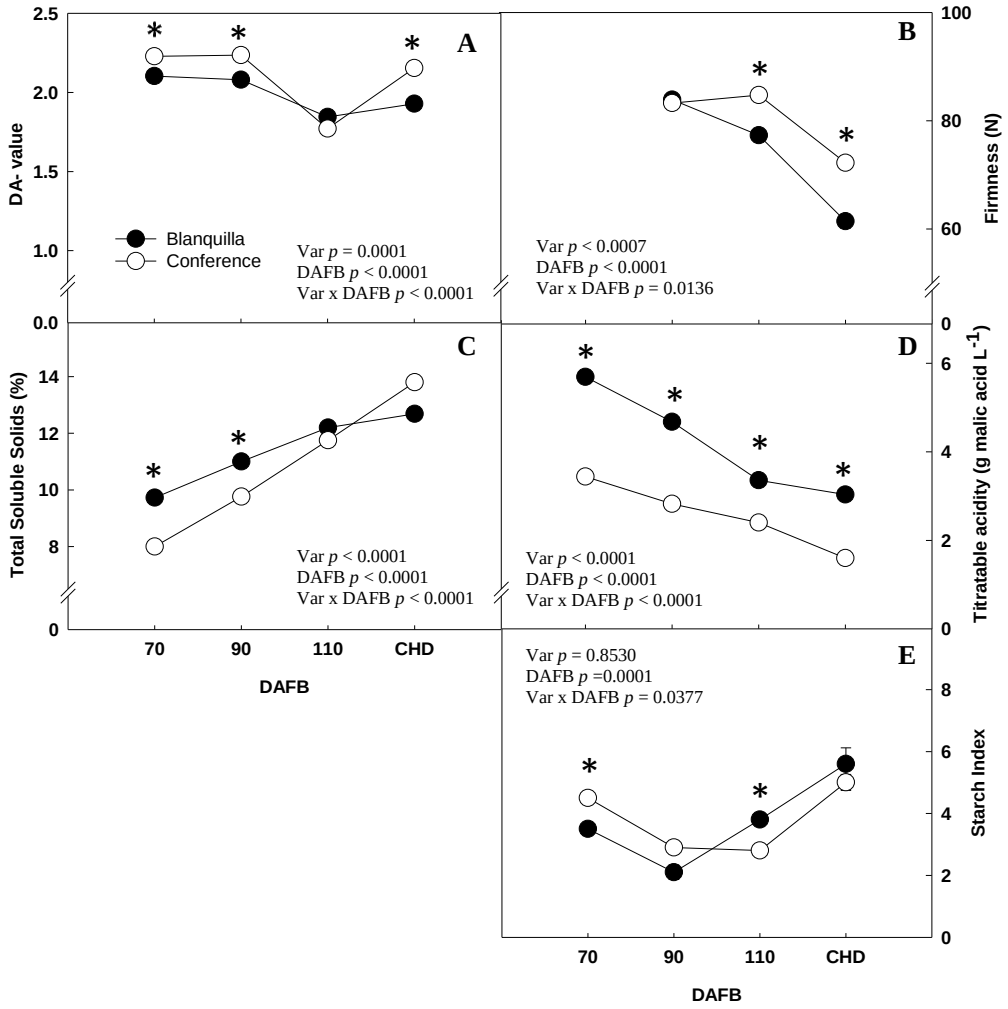
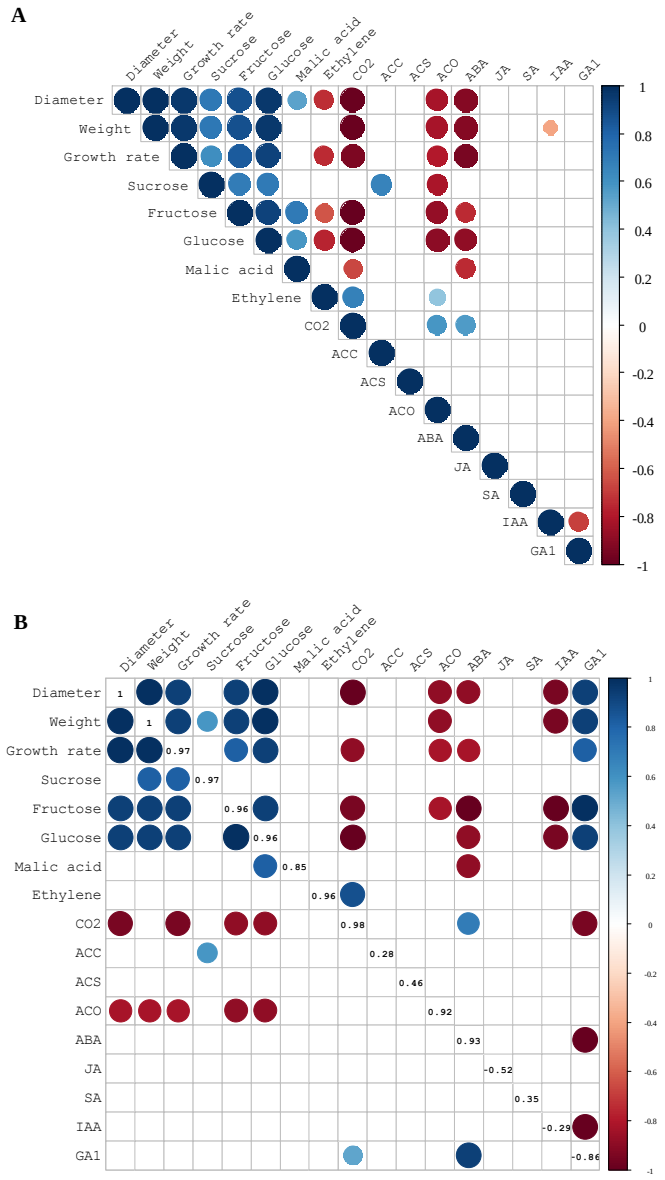
Supplementary data



Supplementary Figure 1: Changes in DA-value (A), firmness (B), total soluble solids (C), titratable acidity (D) and star index (D) in 'Blanquilla' (●) and 'Conference' (○) during different days after full bloom (DAFB). Error bars represent the standard error of the means (n=4). Stars indicate significant differences at $p \leq 0.05$.



Supplementary Figure 2: Visualization of Spearman's rank correlation matrix between quality and biochemical traits for both varieties (A) and for 'Conference' and 'Blanquilla' separately (B; above and below, respectively). Numbers in the diagonal represent the correlations between the same traits among the studied varieties. Circles above and below the diagonal reported the correlation coefficients between traits. Colour intensity of each circle is proportional to the correlation coefficients while the circle size is proportional to the significance level. White squares denote non-significant correlations ($p > 0.05$).

CAPÍTULO 2

New insights on the ripening pattern of 'Blanquilla' pears: A comparison between on- and off-tree ripened fruit

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Postharvest Biology and Technology (2019), https://doi.org/10.1016/j.postharvbio.2018.12.013

Abstract

To better understand the key processes involved in the ripening of attached fruit, we have investigated physicochemical and biochemical changes occurring in 'Blanquilla' pear during on-tree (attached fruit) and off-tree ripening (harvested fruit). Flesh firmness, sugars, acids and the volatile profiles as well as ethylene metabolism, PG and PME enzyme activities and oxidative damage were measured. Firmness loss in detached 'Blanquilla' pear (off-tree) was initially mediated by oxidative stress (higher accumulation of malondialdehyde) and then by ethylene in a process in which 1-aminocyclopropene-1-carboxylic acid (ACC) synthase was the limiting factor. In contrast the progressive but slower softening observed during on-tree fruit ripening was not associated to oxidative damage but rather to a delayed production of ethylene limited, in turn, by the activity of ACC oxidase. An interesting association was found between the initiation of the ethylene production and a concomitant increase of sucrose levels during on-tree ripening also accompanied by a decline in hexanal. The putative role of these compounds as a tree-associated factor modulating on-tree pear ripening is discussed.

Keywords: Ethylene, Hexanal, Oxidative stress, Softening, Sucrose, Tree-factor.

1. Introduction

Fruit have been classified in climacteric and non-climacteric depending on their respiratory and ethylene production patterns during ripening (Paul et al., 2012). In climacteric fruit, an increase in the respiration rate and the ethylene production is observed at the onset of ripening, a phenomenon that is not observed in non-climacteric fruit (Lelièvre et al., 1997). The autocatalytic ethylene production typically allows climacteric fruit to ripen once detached from the tree, whereas non-climacteric fruit do not have this capacity (Van de Poel et al., 2014).

Generally, it is accepted that most European pears, albeit classified as climacteric, are not able to completely ripen on-tree or at the time of commercial harvest unless they received a chilling or ethylene treatment (Villalobos-Acuña and Mitcham, 2008). Depending on the necessity of this chilling period, pears may be grouped into two classes: winter and summer pears. The first ones, include cultivars such as 'Comice' and 'Beurré d'Anjou', require long chilling periods after harvest to produce ethylene and therefore to start the ripening process (Villalobos-Acuña and Mitcham, 2008). That said, the length of the chilling period required to initiate the ripening of winter pears largely differ among cultivars. For instance, 'Beurré d'Anjou' pears may need up to 150 d of cold storage to induce ethylene production whereas 'Packham Triumph' pears may require no longer than 15 d of cold storage (Larrigaudière et al., 2016).

In contrast, summer pears such as 'Rocha' (Saquet and Almeida, 2017), 'Blanquilla' (Larrigaudière et al., 2004) or 'Conference' (Chiriboga et al., 2011) pears require minimum or no exposure to low temperatures to induce this process, as soon as they are harvested at the appropriate maturity. 'Blanquilla' pears also known as 'Spadona di Salermo' in Italy and 'Krystalli' in Greece, is a cultivar capable of ripening both off- and on-tree (Larrigaudière et al., 2004). This specific behaviour makes this cultivar especially suitable to study the biochemical events differentiating off- and on-tree pear ripening.

Earlier studies suggested that the inhibition or delay of on-tree fruit ripening was related to the presence of an inhibiting substance called the 'tree factor' (Abeles, 1973). The 'tree factor' was then thought to be exported from the leaves to the fruit via the phloem, and to affect the fruit ethylene production capacity (Sfakiotakis and Dilley, 1973).

Since then, different hypothesis have been proposed to explain the resistance to ripen on-tree for numerous fruit. The initial hypothesis was based on the climacteric characteristics of the fruit and on the existence of two systems of ethylene production, namely system 1 and system 2 (McMurchie et al., 1972). System 1 is non-autocatalytic and operates in immature fruit whereas System 2 operates during ripening to induce the autocatalytic ethylene production (reviewed in Pech et al., 2008). According to this theory, on-tree ripening impairment is associated to the maintenance of system 1. Klee (2004) suggested that differences between cultivars and in the time of induction of system 2 on-tree depended on the basal levels of ethylene production by system 1 (even if the ethylene levels were low). For others authors, differences between cultivars are related to differences in the fruit sensitivity to ethylene (Biale and Young, 1981; McGlasson, 1985), a sensitivity that is regulated at the receptor level (Kevany et al., 2007).

The regulation of fruit ripening in non-climacteric fruit and by extension, non-detached fruit, is essentially hormonal. Abscisic acid (ABA) was found to play an essential role in strawberry ripening by influencing softening, aroma development and anthocyanin accumulation (Jia et al., 2011, 2013). Jasmonic acid is also involved in strawberry cell wall metabolism (Mukkun and Singh, 2009) and anthocyanin accumulation in apples (Rudell et al., 2002). More recently, Jia et al. (2013) have shown that certain fruit biochemical constituents, such as sucrose also play a key role as a signal involved in strawberry and tomato fruit ripening.

Further studies are still needed to better understand the physiological basis of non-climacteric and on-tree fruit ripening, especially for pears that may ripen or not on-tree. Accordingly, the objective of this study was to compare the ripening behaviour of 'Blanquilla' pears off- and on- the tree. Emphasis was given to monitor changes in global quality traits but also, and especially, on the biochemical and physiological processes explaining these quality changes.

2. Material and methods

2.1. Plant materials and experimental design

'Blanquilla' pear (*Pyrus communis* L.) were harvested on a commercial orchard near Lleida (Catalonia, Spain) at the optimum commercial harvest date (CH; about 125 d after full bloom, DAFB) for the off-tree trial, and 3, 6, 10, 15, 20, 25 and 30 d after commercial harvest (DACH) for the on-tree assay. Off-tree fruit were stored at 20 °C

and 85 % of relative humidity and samples were evaluated at 3, 6, 10, 15 and 20 d. On-tree fruit were harvested and transported to the laboratory each sampling day for immediate analysis.

2.2. Quality evaluations

Flesh firmness was measured on 3 replicates of 6 fruit each per ripening condition with a hand held penetrometer (53200, T.R.Turoni srl., Italy) equipped with an 8 mm probe as described by Chiriboga et al. (2011). Total soluble solids (TSS; %) were measured on pear juice (blend of 6 fruit per replicate and 3 replicates per sampling) using a digital hand-held refractometer (PAL-1, Atago, Tokyo, Japan) whereas titratable acid (TA) concentrations were measured on the same juice samples by titration using 0.1 N NaOH and the results expressed as g malic acid L^{-1}.

The index of absorbance difference ($I_{AD} = A_{670} - A_{720}$) as an indicator of the fruit maturity was measured with a DA-Meter (TR Turoni, Forli, Italy) on opposite sides of the equatorial parts of the fruit.

The starch index was evaluated on 18 fruit samples as described by Almeida et al. (2016) with some modifications. An equatorial slice of each fruit was cut and dipped in a solution of 0.6 % (w/v) iodine in 1.5 % (w/v) potassium iodine for 10 min and then the starch index was subjectively determined using the 10-point scale chart developed by the CTIFL (France). The Streif Index was calculated as [firmness / (SSC * starch index)].

In parallel, flesh tissue from six individual fruit per replicate and three replicates per ripening condition was frozen in liquid nitrogen and kept at −80 °C until further biochemical analysis.

2.3. Ethylene production

Ethylene production (nmol kg^{-1} s^{-1}) was measured as described by Giné-Bordonaba et al. (2017) with some modifications. Four replicates of 3 fruit each were placed in 2 L flasks sealed with a silicon septum for sampling the gas of the headspace after 3 h incubation in an acclimatized chamber at 20 °C. For the analysis of ethylene production, gas samples (1 mL) were taken using a syringe and injected into a gas chromatograph (GC; Agilent Technologies 6890, Wilmington, Germany) fitted with a

FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain) as previously described by Giné-Bordonaba et al. (2014).

2.4. Enzymes related to the ethylene metabolism and fruit softening

1-Aminocyclopropane-1-carboxylic acid oxidase enzyme (ACO) was extracted as described by Chiriboga et al. (2012) with some modifications. The sample (0.5 g of frozen tissue) was homogenized in 1 mL of buffer containing 400 mmol L^{-1} MOPS at pH 7.2, 10 % glycerol, 30 mmol L^{-1} ascorbic acid sodium salt and 2 % PVP 40,000. The homogenate was gently shaken for 10 min at 1 °C and centrifuged at 17,000 *g* for 30 min at 4 °C. Subsequently, the supernatant was stored at −80 °C until analysis.

Enzyme activity was analysed as described in Giné-Bordonaba et al. (2017). The mixture was aired and incubated for 60 min at 30 °C, after which a 1 mL headspace gas sample was injected into a gas chromatograph and the results were expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh weight basis.

The extraction and activity of 1-aminocyclopropane-1-carboxylic acid synthase (ACS) enzyme was determined as previously described by Chiriboga et al. (2013). Briefly, 5 g of frozen tissue were homogenized with 10 mL of extraction buffer containing 200 mmol L^{-1} tricine buffer at pH 8.5, 10 mmol L^{-1} dithiothreitol (DTT), 20 μmol L^{-1} pyridoxal phosphate and 2 % (w/v) PVP. The homogenized was centrifuged at 18,000 *g* for 20 min at 4 °C. Subsequently, 2.5 mL aliquot was loaded into a Sephadex G-25 column (PD 10, GE Healthcare, Buckinghamshire, UK), previously equilibrated with 5 mmol L^{-1} tricine buffer pH 8, 1 mmol L^{-1} DTT and 2 μmol L^{-1} pyridoxal 5-phosphate. The enzyme was eluted with 3.5 mL of the same buffer and 1.5 mL was incubated for 2 h at 25 °C with 200 mmol L^{-1} tricine buffer pH 8 and 100 μmol L−1 of S-adenosyl-L-methionine (SAM). The reaction was then stopped with 100 mmol L^{-1} $HgCl_2$, and 1 mL of the product was mixed and stirred with 100 μL of NaOCl and saturated with NaOH (2:1 v/v). After 2 min, a 1 mL headspace gas sample was injected into a gas chromatograph and the results were expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh weight basis. Pectin methyl esterase (PME; EC 3.1.1.11) enzyme was extracted using the method described by Plaza et al. (2003). PME was extracted by homogenisation of 2 g of frozen ground sample with 6 mL of an extraction solution (1 mol L^{-1} NaCl in 0.2 mol L^{-1} sodium phosphate buffer pH 7.5). The resulting mixture was shaken for 10 min at 4 °C, centrifuged at 16,000 *g* for 20 min at 4 °C and then the

supernatant filtered through six cheesecloth layers. Finally, PME activity from the resulting extract was quantified by titration as described elsewhere (Yeom et al., 2000).

Polygalacturonase (exo-PG; EC 3.2.1.67 and endo-PG; EC 3.2.1.15) extraction and determination was conducted by following the methods described by Van linden et al. (2008). PG activity was calculated as the release of reducing groups per unit of time and per fresh weight ($\mu mol\ kg^{-1}s^{-1}$) based on the two reaction periods as described in Giné- Bordonaba et al. (2017).

2.5. Sugar and organic acid content

Sugars (sucrose, glucose and fructose) and malic acid were extracted from frozen tissue as described by Giné-Bordonaba et al. (2017). The supernatants of each sample extraction were recovered and used for enzyme coupled spectrophotometric determination of glucose and fructose (hexokinase/phosphoglucose isomerase) and sucrose (β-fructosidase) using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer's instructions.

Malic acid was extracted dissolving 2 g of frozen tissue in 5 mL of distillate water. The resulting supernatant from malic extraction was recovered and used for enzyme coupled spectrophotometric determination (L-malate dehydrogenase) of malic acid using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer's instructions.

2.6. Determination of malondialdehyde content

Malondialdehyde (MDA) was analysed as an index of lipid peroxidation using the thiobarbituric acid reactive substrates (TBARS) and according to Martinez-Solano et al. (2005) with some modifications. Briefly, 0.5 g of frozen tissue was homogenized in 4 mL of 0.1 % trichloroacetic acid (TCA) solution. Then, the samples were centrifuged at 24,000 *g* for 20 min at 20 °C and 0.5 mL of the supernatant was added to 1.5 mL of a 0.5 % thiobarbituric acid (TBA) in 20 % TCA solution. Another aliquot (0.5 mL) of the supernatant was added to a solution containing only 20% TCA as a control. The mixture was incubated at 90 °C for 30 min until stopped by placing the reaction tubes in an ice-water bath. Then, the samples were centrifuged at 24,000 *g* for 10 min at 4 °C and the absorbance of the supernatant was read at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The amount of MDA-TBA

complex (red pigment) was calculated using the extinction coefficient 155 L $mmol^{-1}$ cm^{-1} and the results expressed as μmol kg^{-1} on a fresh weight basis.

2.7. Volatile determination by SPME-GC-FID

The extraction and concentration of volatile compounds was done as described by Qin et al. (2012) with some modifications. A SPME fibre with 65-μm layer of polydimethylsiloxane–divinylbenzene (65 μm PDMS/DVB; Supelco Co., Bellefonte, PA, USA) was used and activated before sampling according to the manufacturer's instructions. For each extraction, 10 g of frozen tissue was placed into a 20-mL vial containing 3.6 g of NaCl to facilitate the release of volatile compounds. Before sealing the vial, 2 μL of 32 μL L^{-1} 3-nonanone was added as internal standard. A magnetic follower was added to each vial, which was placed into a constant-temperature water bath at 40 °C with stirring. Samples were equilibrated for 20 min and then the SPME fibres were exposed to the headspace of the sample for 30 min to adsorb the volatiles. The volatile compounds were subsequently desorbed over 10 min at 240 °C into the splitless injection port of the chromatograph. The volatile constituents were identified and quantified with an HP 6890 A gas chromatograph with a flame ionization detector equipped with a capillary column with cross-linked free fatty acids as the stationary phase (FFAP; 50m × 0.2 mm × 0.33 mm). Helium was used as the carrier gas at a constant flow of 1.0 mL min^{-1}. The injector and detector temperatures were 240 °C. The oven temperature programme was 35 °C for 8 min, increasing at 2 °C min^{-1} to 140 °C and holding for 2 min, then increasing at 10 °C min^{-1} to 240 °C and holding for 5 min. Compounds were identified by comparing their respective retention index with those of standards. All of the standards for the volatile compounds studied in this work were analytical grade or the highest quality available. Quantification was performed using individual calibration curves for each compound. The concentrations of volatile compounds were expressed as μg kg^{-1} on a fresh weight basis.

Compound identification was performed on an Agilent 6890 N gas chromatograph/mass spectrometer (Agilent Technologies, Inc.) using the same capillary column as used in the GC analyses. Mass spectra were obtained by electron impact ionization at 70 eV. Helium was used as the carrier gas, and the same temperature gradient programme described previously was used for MS acquisition. Spectrometric data were recorded (Hewlett-Packard 3398 GC Chemstation) and compared with those from the original NIST HP59943C library mass spectra.

2.8. Statistical analysis

All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS Institute Inc. Mean comparisons for the interaction ripening condition * day was evaluated using Tukey's test at a significance level of $p \leq 0.05$, while comparisons between ripening conditions at specific days was done by least significant difference values (LSD; $p \leq 0.05$) using critical values of t for two-tailed tests for the rest of parameters. A principal component analysis (PCA) was also performed to characterize the samples according to their volatile profile, quality parameters and biochemical traits. The samples included in the PCA were: On-tree 0 d, On-tree 6 d, On-tree 25 d and Off-tree 6 d. A total of 33 variables (26 volatile compounds, 3 quality parameters (firmness, titratable acidity and total soluble solids) and 4 biochemical traits (sucrose, glucose, fructose and malic acid)) were used to perform the data matrix. Data were centred and weighted using the inverse of the standard deviation of each variable in order to avoid the influence of the different scales used for the variables. All analyses were carried out using the PCA platform of JMP® 13.1.0 SAS Institute Inc.

3. Results and discussion

3.1. Changes in overall quality during on-tree and off-tree ripening

The flesh firmness of 'Blanquilla' pear at harvest was 60 N, which was inside the commercial harvest range for this pear cultivar (Gamrasni et al., 2010). After harvest, firmness from off-tree fruit decreased rapidly from days 3 to 6 (*ca.* -11.2 N d^{-1}) and then more gradually until reaching 5 N at 20 d. In contrast, the loss of firmness in attached fruit started 10 d later than that observed in detached fruit and was more progressive, reaching 5 N only after more than 30 d (Fig. 1A).

TSS of the pears at harvest was 13 % (Fig. 1B), thereby similar to the results obtained in previous studies on other European pear cultivars like 'Jules d'Airolles', 'Abate Fetel' and 'Spadona'(Gamrasni et al., 2010; Yim and Nam, 2016). No differences in TSS were observed between off- tree and on-tree fruit until 5 d. Then, TSS was higher in off-tree samples than in those fruit ripened on-tree.

No significant differences were found for TA between off-tree and on-tree fruit (Fig.1C). TA at harvest was 4.10 ± 0.10 g malic L^{-1} and then steadily decreased (-0.07 g L^{-1}) until reaching values of *ca.* 2 g L^{-1} both for off-tree and on-tree fruit. TA

values in this work were slightly higher than those observed in previous studies with 'Blanquilla' (Larrigaudière et al., 2004) for which TA values were always lower than 3 g L^{-1}. Different orchards or agroclimatic conditions may explain the differences in the TA values between both studies.

The SI at harvest was almost 6 (Fig. 1D) and then gradually increased until reaching values of completely mature fruit (SI = 10). The highest SI in off-tree fruit was reached 10 d after storage at 20 °C, whereas on-tree ripened fruit needed 25 d to reach the same value. The slower starch degradation during on-tree ripening may be explained by: (1) the differences in the ethylene production kinetics (Fig. 2A), since it is well documented that starch degradation is for some pome fruit cultivars an ethylene-related phenomena (Thammawong and Arakawa, 2007) and a good indicator of the fruit maturity stage (Peirs et al., 2002); but also by (2) the continuous supply of carbohydrates from source-to-sink occurring in attached fruit and satisfying the fruit needs for respiration and other catabolic process.

Chlorophyll degradation measured by the index of absorbance difference I_{AD} (Fig. 1E), as well as the Streif index (Fig. 1F), also reflected the slower ripening pattern of attached fruit compared with detached fruit and highlighted the suitability of the former non-destructive measurement as a potential tool to determine the optimal harvest date in 'Blanquilla' pears. However, this tool less accurately represented quantitative differences, not only in firmness loss but in other quality attributes, and was less useful to follow the ripening process during off- tree ripened fruit.

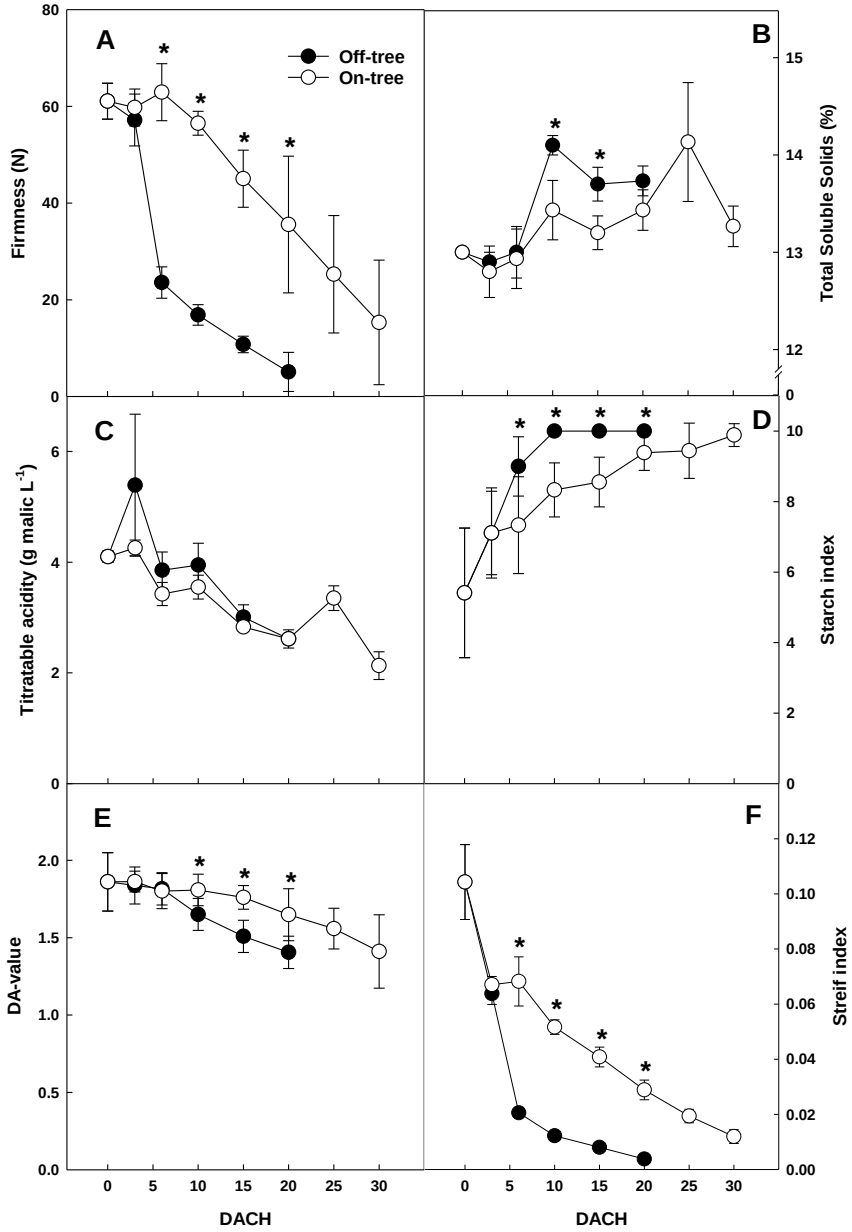


Figure 1. Changes in fruit firmness (A), total soluble solids (B), titratable acidity (C), starch index (D), DA-value (E) and Streif index (F) during off-tree (●) and on-tree (○) ripening. DACH stands for Days After Commercial Harvest. Error bars represent the standard deviations of the means (n=3). Stars indicate significant differences at $p \leq 0.05$.

3.2. Ethylene metabolism and its regulation on- and off-tree

Differences in the kinetics of ethylene production were found between off-tree and on-tree ripened fruit (Fig. 2A). Harvested fruit (off- tree) exhibited a typical pre-climacteric behaviour with a delay (5–6 d) in the initiation of ethylene production and then a sharp increase up to 0.12 nmol kg^{-1} s^{-1} at 20 DACH. The kinetic of ethylene production for on-tree fruit was much more progressive, with an extension of the lag period up to 10 d and a slower ethylene production rate, being nearly half of that observed in fruit ripened off-tree (Fig. 2A). Temperature conditions among on- and off-tree ripened fruit were relatively similar (Supplementary Fig. 1) and did not explain the differences in ethylene production. However, it is also likely that warmer temperatures during on-tree ripening may lead to higher ethylene production, yet not reaching similar values to those observed in detached fruit. The ability of 'Blanquilla' pear to produce ethylene at relatively high levels on-tree is atypical in pears. Only one study have shown a similar tendency in 'La France' pears which needed up to 14 d on-tree to produce 1 μL of ethylene kg^{-1} h^{-1} (equivalent to 0.01 nmol kg^{-1} s^{-1}, 10- fold lower values; Murayama et al., 1998). However, similar trends have been reported in 'Gala' (Lin and Walsh, 2008) and other apple cultivars. Others summer pear cultivars, such as 'Conference', are more resistant to produce ethylene and are more difficult to ripen, even off- tree, if harvested when slightly immature (Chiriboga et al., 2011).To better understand the specific behaviour of 'Blanquilla' pear regarding ethylene production, we analysed the changes in ACC metabolism and more specifically the changes in the activity of the enzymes ACS and ACO both off- and on-tree. The differences of ethylene production between on- and off-tree samples (Fig. 2A) were not exclusively explained by differences in ACS enzyme activity (Fig. 2B). In off-tree samples, ACS activity remained inactive for 3 d and sharply increased thereafter until day 6. In on-tree fruit, a steady increase in the ACS activity was observed throughout the different samplings. ACO activity in off-tree fruit was higher than on- tree (Fig. 2C), increasing immediately after harvest and reaching a value of 0.17 nmol kg^{-1} s^{-1} at 3 DACH. Overall, our results suggest that ACO and ACS act differentially as limiting factors for ethylene production during on- and off-tree ripening, respectively. ACO in on-tree ripened fruit was activated only after 10 d, parallel to the increase in the ethylene production rate (Fig. 2A and C). These findings support the theory of the 'tree factor' (Abeles, 1973) where it was hypothesized that the 'tree factor' is an inhibitor of ethylene production exported from the leaves to the fruit via the phloem (Sfakiotakis and Dilley, 1973). This inhibitor is thought to affect System 2 ethylene production (Lin and Walsh, 2008) and its action may be inhibited by defoliation and

girdling techniques (Sfakiotakis and Dilley, 1973). Our results are in accordance with the 'tree factor' theory and with the putative presence of an inhibitor on-tree. With this in mind, we analysed the differences in assimilates accumulation (sugars and acids) and changes in the fruit volatiles during off- and on-tree fruit ripening.

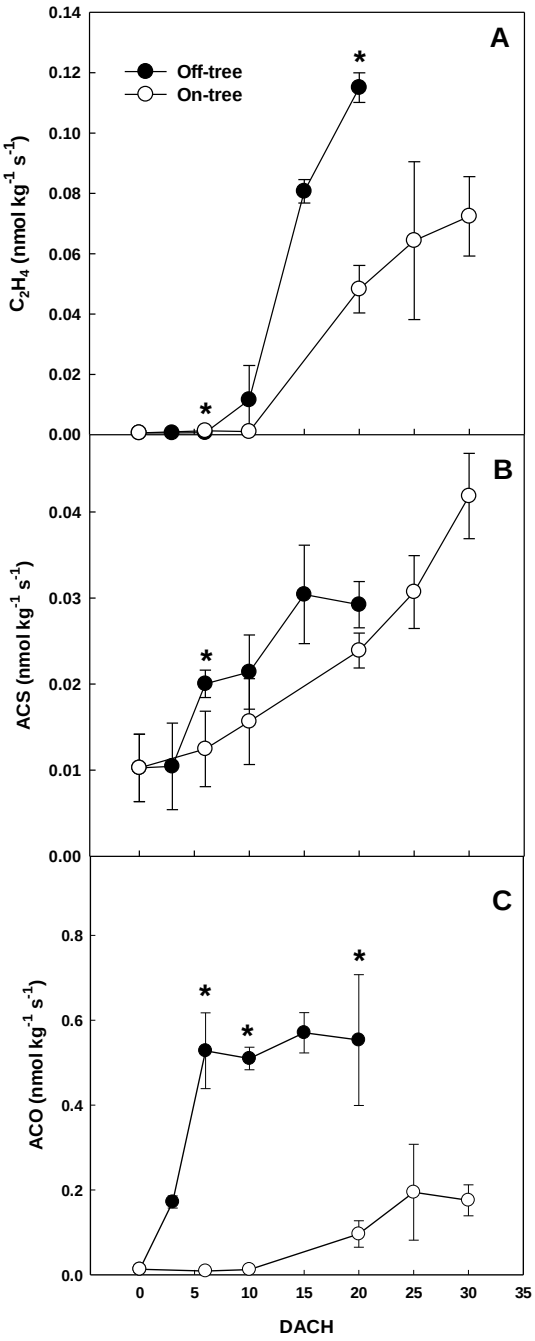


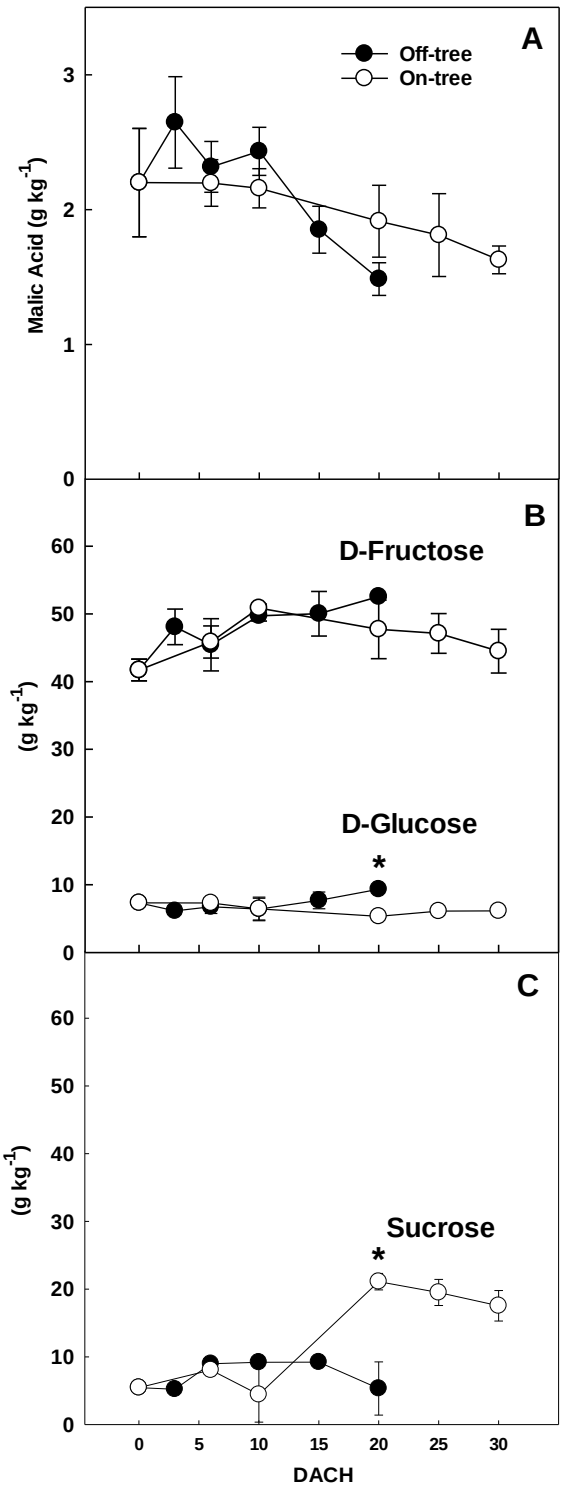
Figure 2. Changes in ethylene production (A), ACC synthase activity (B) and ACC oxidase activity (C) in off-tree (●) and on-tree (○) ripening. DACH stands for Days After Commercial Harvest. Error bars represent the standard deviations of the means (n=4 for ethylene production and n=3 for ACC and ACS activity). Stars indicate significant differences at $p \leq 0.05$.

3.3. Are assimilates involved in the regulation of on-tree pear ripening?

Malic acid content did not differ between the fruit ripened on-tree and off-tree and generally decreased from *ca.* 2 to 1.5 g kg^{-1} through storage at 20 °C or ripening on the tree (Fig. 3A). Faster utilisation of malate in off-tree ripened fruit was observed from day 10 onwards if compared to fruit ripened on-tree. The decreased in malic acid off-tree was paralleled by changes in DA-values, maximum starch index and opposite to the rise of the ethylene. This pattern may be easily explained by the fact that malic acid is a respiratory substrate and it is probably used by pear fruit as the carbon source in the tricarboxylic acid cycle (Ma and Chen, 2003). Albeit no information is readily available for pears, in grapes, malic acid is thought to be an important respiratory substrates (Famiani et al., 2014) and postharvest studies on apples also point out the importance of this compound in fruit respiration (Liu et al., 2016).

Only slight differences in glucose accumulation were observed between off- and on-tree fruit until 10 d (Fig. 3B). Afterwards, the glucose levels slightly increased in off-tree ripened fruit (up to 10 g kg^{-1}), whereas remained at a constant value of 5 g kg^{-1} in samples ripened on-tree. In both on- and off-tree, fructose was the predominant sugar with concentrations ranging from 40 to 50 g kg^{-1} (Fig. 3B). Accordingly, fructose, sorbitol, sucrose and, in lower amount, glucose are known to be the major sugars in pears (Barroca et al., 2006). In off-tree samples, sucrose levels decreased between 15 and 20 DACH (Fig. 3C) in parallel to a slight increases in glucose and fructose levels. This behaviour has also been described by Itai et al. (2015) in 'Gold Nijisseiki' pear. In contrast, the sharp increase in sucrose levels observed in attached fruit after 10 d (Fig. 3C) was not related to changes in glucose and fructose content but coincident with the induction of the ethylene burst and to the initiation of the ripening process. This last result is of interest and shows, in agreement with the results described by Murayama et al. (2015) or Kim et al., (1987) that sucrose or galactose may act as a signal molecule for on-tree fruit ripening. Indeed, previous studies have also shown that galactosyl compounds stimulate C_2H_4 production in tomato (Kim et al., 1987).

There is clear evidence that sucrose may play a pivotal role in different processes of plant biology such as for instance the signalling of assimilates partitioning (Chiou and Bush, 1998) or the induction of anthocyanin biosynthesis (Teng et al., 2005). It is also recognized that sucrose plays an important role in the regulation of tomato (climacteric) and strawberry (non-climacteric) fruit ripening (Jia et al., 2016, 2013).



Our results further suggest a pivotal role of this sugar in pear ripening and especially when understanding the capacity to ripening on-tree. Although on-tree pear fruit cannot be considered as non-climacteric fruit model, the overall physiological changes observed in attached 'Blanquilla' pears, and especially regarding ACO, let us to hypothesize that on-tree pear ripening may be regulated by similar effectors than those controlling non-climacteric fruit ripening. Accordingly, sucrose may act as an important regulatory factor of 'Blanquilla' pear ripening on-tree. Further studies are needed to confirm these results and especially to determine the role that sucrose or its interplay with other key hormones (i.e. abscisic acid) may have on regulating on-tree ripening.

Figure 3. Changes in malic acid content (A), D-Glucose and D-fructose levels (B) and sucrose levels (C) during off-tree (●) and on-tree (○) ripening. DACH stands for Days After Commercial Harvest. Error bars represent the standard deviations of the means (n=3). Stars indicate significant differences at $p \leq 0.05$.

3.4. The physiological basis of firmness loss both on- and off-tree in relation to cell-wall degrading enzymes and oxidative stress

It is interesting to note the lack of a relationship observed between the ethylene production and softening for off-tree ripened fruit. Loss of firmness was observed after 3 d of ripening at 20 °C (Fig. 1A), while detectable ethylene production started only after 5 d (Fig. 2A). Since firmness loss is assumed to be an ethylene dependent process, such atypical behaviour in 'Blanquilla' pear remains to be clarified.

Hence, in an attempt to further understand the softening pattern of 'Blanquilla' pear we investigated the activity of some cell-wall degrading enzymes including PME and PG which are thought to be ethylene-dependent (Pech et al., 2008). No differences in PG activity were observed between off-tree and on-tree ripened fruit (Table 1). PG activity remained at a constant levels both on- and off-tree indicating that this enzyme is likely ethylene-independent in 'Blanquilla' pear. In other studies, it has also been demonstrated that initial fruit softening (i.e. in tomato) is associated with a decline in some cell wall components without increased PG activity (Gross and Wallner, 1979). Only slight differences between on- and off-tree samples were found for PME (Table 1), suggesting also that this enzyme may not have a pivotal role in 'Blanquilla' pear softening. These results are consistent with those observed in 'Golden Reinders' apples (Ortiz et al., 2011) and also in tomato (Tieman and Handa, 1994) where PME activity did not play a key role on fruit softening.

Table 1: PG (nmol kg^{-1} s^{-1}) and PME (μmol kg^{-1} s^{-1}) levels in 'Blanquilla' pear during off-tree and on-tree ripening. Means ± standard deviation followed by the same letter are not significant different at $p \leq 0.05$ (n=3).

PG (nmol kg^{-1} s^{-1})			PME (μmol kg^{-1} s^{-1})		
DACH	Off-tree	On-tree	DACH	Off-tree	On-tree
0	19.38±2.052 a	19.38±2.052 a	0	6.64±1.838 bc	6.64±1.838 bc
3	18.15±2.613	-	3	5.20±0.797	-
6	16.47±1.385 b	20.18±1.871 ab	6	4.52±1.019 cd	10.36±0.207 a
10	18.06±2.937 ab	17.91±0.418 ab	10	5.23±0.759 bcd	11.07±1.639 a
15	17.84±1.182	-	15	5.29±0.906	-
20	18.08±2.167 ab	23.09±1.708 a	20	8.84±0.323 ab	3.60±0.738 cd
25	-	18.30±1.632	25	-	5.67±1.108
30	-	17.44±0.764	30	-	2.72±0.955

Since differences in the softening pattern between on-tree and off- tree samples were not explained by cell-wall degrading enzymes nor by ethylene metabolism, we hypothesized that such differential pattern could be mediated by oxidative stress. Accordingly, we measured the MDA contents (Fig. 4A), a typical marker of oxidative stress resulting from lipid peroxidation.

At harvest, the concentration of MDA was about 17 μmol kg^{-1}. In off-tree samples, this concentration increased up to 30 μmol kg^{-1} during the first week of storage. At the same time, ethylene production remained low and the fruit lost more than half of their initial firmness. These results clearly suggest that initial firmness loss (from 3 to 6 DACH) in off-tree 'Blanquilla' pear was not ethylene dependent but rather associated to oxidative stress. This process may also be related to the 'water-stress' phenomena experienced by detached fruit, which in turn may be linked to ABA. A strong negative correlation was observed between the MDA content and firmness (Fig. 4B). A similar behaviour was also observed in Japanese pear (Li and Wang, 2009) for which an 8-fold increase in MDA content was described after 6 d at room temperature if compared to the values at harvest.

In contrast to off-tree fruit, MDA levels in on-tree ripened fruit only significantly increased after 25 d and regardless of the changes in ethylene production and firmness loss. Overall, these results suggest that oxidative stress was unlikely involved in the firmness loss observed after 10 d and in the induction of the ripening process in fruit ripened on-tree. As described earlier, and in contrast to off-tree ripened fruit, on-tree fruit softening appeared to be exclusively ethylene dependent. Collectively these results are of interest and highlight clear differences in the susceptibility of off- and on-tree fruit to oxidative stress that likely determine the initiation of fruit ripening.

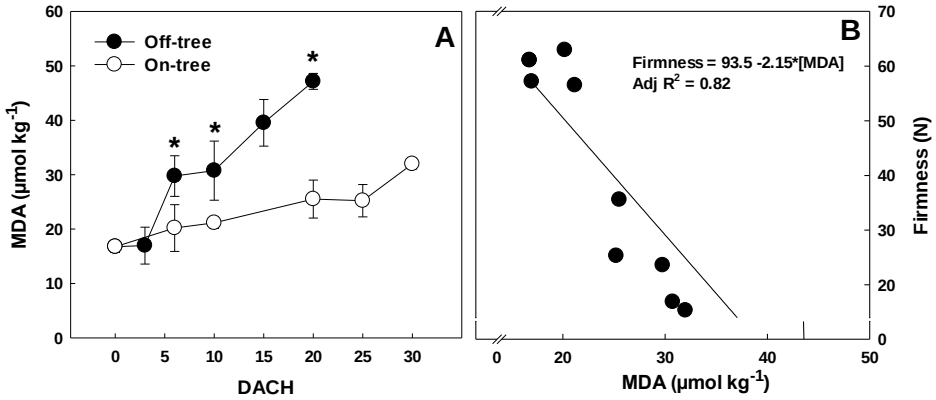


Figure 4. Changes in the concentration of malondialdehyde (A) and the correlation between firmness and MDA content (B) during off-tree (●) and on-tree (○) ripening. DACH stands for Days After Commercial Harvest. Error bars represent the standard deviations of the means (n=3). Stars indicate significant differences at $p \leq 0.05$.

3.5. Changes in the volatile profile during on and off-tree ripening

Pears are highly appreciated by consumers due in part to their unique and complex aroma profiles associated to each specific cultivar (Chen et al., 2018) but also to its characteristic ripening process.

Accordingly, we investigated whether ripening off- tree impaired or enhanced the development of the 'Blanquilla' pear volatile profile. Fifteen compounds belonging to different chemical classes: esters (8), aldehydes (2), alcohols (4) and terpene (1) were identified (Table 2). The predominant compounds in all the samples with concentrations higher than 1500 µg kg^{-1} were hexanal, butyl butanoate, and α-farnesene, as found for 'Yali' pear (Chen et al., 2006). The presence and abundance of volatile compounds on fruit ripened off-tree for 6 d was similar to that observed on fruit ripened on-tree for 25 d, both of them characterised with similar firmness values. No significant differences in the concentration of any volatile compound were found between these samples, except hexyl acetate, which was almost 3-fold higher in fruit ripened off-tree for 6 d. In the case of ripe fruit (on-tree 25 d and off- tree 6 d), 4 straight esters (propyl acetate, butyl acetate, pentyl acetate and hexyl acetate) and one alcohol (1-butanol) were the majority compounds. 1-Butanol was present in all the samples analysed being *ca.* 20-fold higher in ripe than in unripen fruit (0 DACH). These results are in accordance with previous studies in 'Bartlett' pears, where 1-butanol concentration drastically increased during ripening (Zlatić et al., 2016). In addition, it

has been shown that completely ripe pear have a higher concentration of esters than firmer ones (Makkumrai et al., 2014), so this may explain the fact that the majority of esters in our samples were present in fruit ripened on-tree for 25 d and in off-tree for 6 d yet not at the time of commercial harvest. Hexanal was the principal aldehyde and there were no significant differences neither between the content in immature fruit (on-tree 0 d and on-tree 6 d), nor between ripe fruit samples (off-tree 6 d and on- tree 25 d). In contrast, there were significant differences for this compound when comparing different DACH, with hexanal content declining as fruit ripened off-tree but also on-tree. In on-tree ripened fruit (25 DACH), hexanal contents were nearly half than that observed in fruit at the time of commercial harvest. Similar results were obtained by Makkumrai et al. (2014) in 'Bartlett' pear, where hexanal also decreased as the fruit ripened. Interestingly, hexanal contents remained high during the first days of on-tree fruit ripening, when no softening occurs, pointing out a potential role of this compound to modulate on- tree ripening process of 'Blanquilla' pear. Exogenous application of this compound are known to inhibit fruit ripening (Pak Dek et al., 2018) by decreasing transcript levels of phospholipase D and other ripening-related genes.

Table 2: Average concentrations of volatile compounds. Means ± standard deviation followed by the same small letter indicate no significant differences among the control and on-tree samples for each compound. Means ± standard deviation followed by the same capital letter indicate no significant differences between the samples on-tree 25 d and off-tree 6 d for each compound ($p \leq 0.05$; n=3). nd = non detected.

Volatile compounds (µg kg^{-1})	**On-tree**			**Off-tree**
	0d (61.1 N)	6d (62.9 N)	25d (25.3 N)	6d (23.6 N)
Ethanol	nd	nd	0.46±0.158 A	0.22±0.025 A
Propyl acetate	nd	nd	543.36±332.428 A	372.10±108.541 A
Butyl acetate	nd	nd	8620.90±2668.309 A	16953.53±5685.289 A
Hexanal	18080.67±1344.572 a	19547.21±2445.170 a	10635.48±4289.035 bA	7897.73±2053.258 A
Butanol	23.77±10.561 b	17.17±2.011 b	375.36±123.527 aA	415.84±103.391 A
Pentyl acetate	nd	nd	844.64±158.209 A	1363.10±582.786 A
2-Methyl-1-Butanol	177.12±19.420 ab	200.60±54.486 a	95.51±25.378 bA	68.82±10.577 A
Butyl butanoate	3921.15±1387.091 a	2653.79±998.638 a	3041.76±1068.421 aA	4194.76±501.560 A
Hexyl acetate	nd	nd	9435.01±112.496 B	24294.35±3825.698 A
Hexyl propanoate	nd	nd	50.00±10.156	nd

3.6. Exploring the organoleptic changes occurring during on- and off-tree pear ripening

To further explore the relationship between the taste-related (individual sugars, malic acid, TSS and TA) and the volatile composition of fruit ripened on- and off-tree, we performed a multivariate analysis. A principal component analysis (PCA) was carried out to assess differences between on-tree and off-tree samples or among the different days on-tree. Two principal components 1 (PC1) and 2 (PC2) were sufficient to explain 75.7 % of total variability of the samples (Fig. 5). There were three well-separated groups: one to the left of the plot corresponding to the samples on-tree 0 d and on-tree 6 d, a second group on the top right of the plot corresponding to samples ripened on-tree for 25 d, and the third group located in the middle down corresponding to the samples ripened off-tree for 6 d. The highest emissions of α-farnesene, 2-methyl-1-butanol, octyl acetate, hexanal and butyl hexanoate, together with high firmness and high glucose concentration, were found for fruit harvested at the optimum commercial date (0 DACH) but also for fruit ripened on-tree for 6 d (more immature fruit). Conversely, fruit let attached on the tree for 25 d, showed the lowest emissions of these variables along with the highest emission of ethanol, hexyl propanoate, propyl acetate, butanol, benzyl alcohol, together with high concentrations of sucrose, fructose and total soluble solids (TSS). The last group was related to the samples ripened off-tree for 6 d which was characterised by including those samples with higher amounts of butyl, hexyl and pentyl acetate, all of these compounds being previously identified as primary contributors to pear aroma (Suwanagul et al., 1998). Overall, and despite the lack of significant differences when considering absolute values, on-tree ripened fruit have a distinct volatile blend and physicochemical characteristics than fruit ripened off-tree. Future studies should address if such differences can result in different consumer preferences.

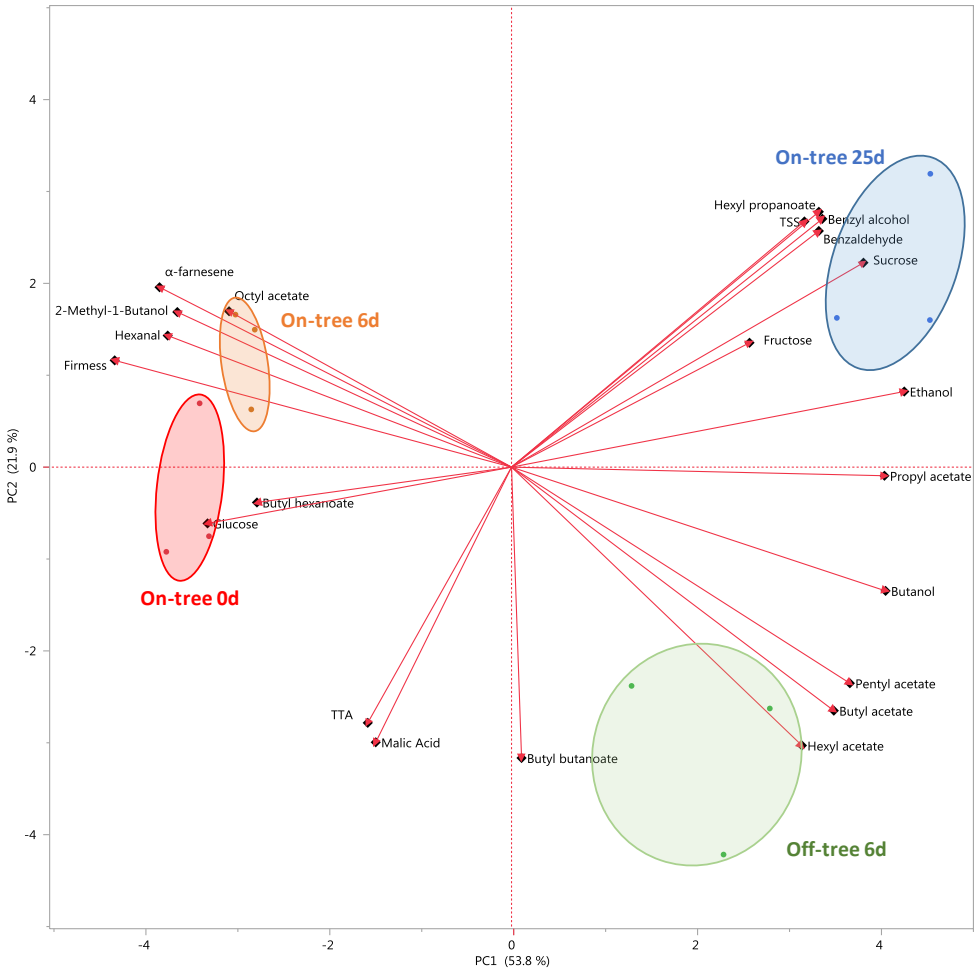


Figure 5. Principal components analysis of volatile profile, quality parameters and biochemical traits in control fruit (CHD = 0 d on-tree), fruit ripened 6 d on-tree, fruit ripened 25 d on-tree and fruit ripened 6 d off-tree.

4. Conclusions

The results from this study provide new information on the biochemical events differentiating on-tree and off-tree 'Blanquilla' pear ripening. In both samples, ACC metabolism plays a key role yet under different regulatory mechanisms. In off-tree pears, ripening (ethylene production and softening) is initially regulated by oxidative stress that likely promotes the further autocatalytic burst of ethylene production through an activation of ACS. In contrast, no oxidative stress was detected in on-tree fruit, where ripening seems to be regulated by ACO and initially inhibited by hexanal,

but also, in the later stages of ripening, by the accumulation of sucrose possibly triggering the initiation of ethylene production.

Future studies are required to better understand the role that ethylene, volatiles and sucrose or its interplay with other crucial hormones such as ABA may have in the ripening process of pear fruit, and especially in other pear cultivars that do not have the capacity to ripen on-tree. The results from this study may provide a better understanding of the ripening process in attached pears hence making easier the decisions for optimal harvest in terms of fruit quality.

5. Acknowledgments

This work was supported by the CERCA Programme/ Generalitat de Catalunya and by the Ministerio de Economía y Competitividad / MINECO (grant AGL2017-87923-R). Thanks are given to Dolors Ubach and Elisabet Duaigües for technical support.

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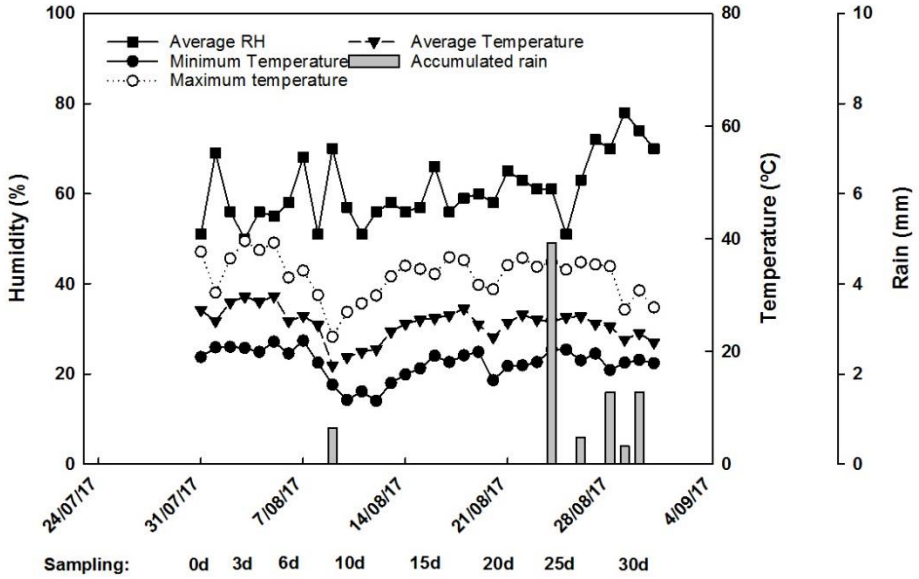
Supplementary data



Supplementary Figure 1: Temperature, relative humidity and rainfall during the period between samplings for on-tree ripened fruit. Off-tree ripened fruit were stored at 20 ± 0.5 °C and 85 % RH.

CAPÍTULO 3

Elucidating the involvement of ethylene and oxidative stress during on- and off-tree ripening of two pear cultivars with different ripening patterns

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Plant Physiology and Biochemistry (2020) https://doi.org/10.1016/j.plaphy.2020.08.018

Abstract

Scarce information is available about the ripening process of European pears attached and detached from the tree. Accordingly, this study aimed to investigate the physiological and biochemical processes underlying both on- and off-tree fruit ripening in a summer ('Conference') vs. a winter ('Flor d'Hivern') pear cultivar. For each cultivar, a batch of fruit was harvested at the commercial harvest date and ripen at 20 °C and another batch was left to ripen on the tree. In both cultivars the inability of the fruit to soften on-tree, was related to a very limited ethylene metabolism but also associated to high content of H_2O_2 and low lipid peroxidation levels. In contrast, ripening in detached fruit was cultivar-dependent. In 'Conference' pears, the sharp firmness loss and colour changes observed during off-tree ripening were not strictly associated to an enhanced ethylene production but rather triggered by an oxidative related process preceding the climacteric rise. In contrast, 'Flor d'Hivern' pears experienced limited softening and degreening during off-tree ripening not being related to the action of ethylene or oxidative stress. Collectively our results showed that pear ripening was not exclusively dependent of ethylene production and that the fruit potential to limit oxidative damage may be involved with the inability of some European pear cultivars to ripen on-tree.

Keywords: 1-aminocyclopropane-1-carboxylic acid metabolism, H_2O_2, malondialdehyde, *Pyrus communis*, ripening

1. Introduction

Climacteric fruit are characterised by an increase in ethylene production and respiratory rate at the onset of ripening and the ability to ripen once detached from the plant (Lelièvre et al., 1997). European pears (*Pyrus communis* L.) are usually classified as climacteric fruit even though several cultivars are not capable to ripen normally after harvest unless receiving an ethylene or chilling treatment (Villalobos-Acuña and Mitcham, 2008). Thus, European pears are generally divided into summer or winter pears depending on their chilling requirements to achieve normal ripening (Saquet and Almeida, 2017). Summer pears require a minimum or no cold storage period to ripen normally and to produce ethylene after harvest. In contrast, winter pears need medium to long exposure to low temperatures to initiate the autocatalytic ethylene and thereby ripen (Villalobos-Acuña and Mitcham, 2008). Differences between summer and winter pear cultivars are also reflected during on-tree ripening, since the latter group would generally experience little or no firmness loss when left to ripen on the tree (Lindo-García et al., 2020a; Murayama et al., 1998).

A recent study on a typical summer pear cultivar ('Blanquilla') has shown that not only an enhanced ACC oxidase enzyme activity but higher sucrose content were likely modulating the capacity of this pear cultivar to ripen even on-tree (Lindo-García et al., 2019). Indeed, evidence exists suggesting that sucrose, in combination with other compounds, may be involved in the regulation of fruit development in both non-climacteric (i.e. strawberries) and climacteric fruit (Jia et al., 2013). Whether an impairment in ethylene biosynthesis or an altered sucrose metabolism may be responsible for the inability of winter pears to ripen on-tree is still unknown.

Strong evidences also suggest that not only ethylene but other hormones are involved in the regulation of fruit development and ripening (Kumar et al., 2014; Lindo-García et al., 2020b; McAtee et al., 2013), and that the hormonal cross-talk may determine the capability of some fruit to ripen or not on-tree or once detached. In pears, it was proposed that gibberellins, and especially gibberellin 1, are likely acting as ripening inhibitors (Lindo-García et al., 2020b), thereby explaining the inability of the fruit to ripen and to produce ethylene when still attached to the tree. Other authors have shown that a decrease in auxin levels initiates the ripening process in 'Bartlett' pears and regulates the fruit's responsiveness to ethylene (Nham et al., 2015). Whether these specific hormones or other compounds may be considered as the 'tree factor' and

account for the observed resistance to ripening on-tree, is still debatable but undoubtedly warrants further studies.

Accordingly, the aim of this study was to investigate the major physiological and biochemical changes accompanying the ripening of a summer ('Conference') vs. a winter-type ('Flor d'Hivern') pear both on- and off-tree. Emphasis has been given on ACC metabolism but also on fruit oxidative behaviour and changes in assimilate levels to better understand the determining factors involved in the hypothesized 'tree factor' in pears.

2. Material and methods

2.1. Plant materials and experimental design

'Conference' and 'Flor d'Hivern' pears (*Pyrus communis* L.) were harvested from a commercial orchard near Lleida (Catalonia, Spain). 'Conference' pear was selected as a model for summer pear while 'Flor d'Hivern' is a local cultivar, which does not produce ethylene even after long periods of cold storage, behaving like a winter pear type (Lindo-García et al., 2020a). At the commercial harvest date (CHD), fruit (n=174 per cultivar) were randomly harvested from 15 trees and stored in acclimatised chamber at 20 °C and 90 % relative humidity. Off-tree samples were evaluated at harvest and after 3, 7, 15, 21 and 28 d after commercial harvest (DACH). For the on-tree assay, fruit (n = 27 per sampling and cultivar) were randomly taken from 6 different trees and evaluated at the same sampling days than off-tree fruit. All fruit were taken from a similar position within the canopy and transported to the laboratory for immediate analysis as follows. Metereological data for the duration of the on-tree ripening period was retrieved from an agrometereological station located 2 Km away of the experimental orchard.

2.2. Quality evaluations at harvest

Flesh firmness (N) was measured on 3 replicates of 5 fruit each per ripening condition with a penetrometer (T.R.Turoni srl., Italy) equipped with an 8 mm probe as described by Chiriboga et al. (2011). Total soluble solids (TSS; %) were measured on pear juice (blend of 5 fruit per replicate and 3 replicates per sampling) using a digital hand-held refractometer (Atago, Tokyo, Japan) whereas titratable acidity (TA) was measured on the same juice samples by titration using NaOH 0.1N and the results expressed as g malic acid L^{-1}.

The index of absorbance difference (I_{AD} = A_{670} – A_{720}) as an indicator of the fruit maturity was measured with a DA-Meter (TR Turoni, Forli, Italy) on opposite sides of the equatorial parts of the fruit. In parallel, degreening was evaluated by visual inspection in 15 fruit in order to assess the colour turn during the ripening process

The starch index (SI) was evaluated on 15 fruit samples as described by Almeida et al. (2016) with some modifications. An equatorial slice of each fruit was cut and dipped in a solution of 0.6 % (w/v) iodine in 1.5 % (w/v) potassium iodine for 10 min and then the starch index was subjectively determined using the 10-point scale chart developed by the CTIFL (France).

In parallel, two equatorial flesh slices covering all the fruit (avoiding the core fruit) from four individual fruit per replicate and three replicates per ripening condition were frozen in liquid nitrogen and kept at -80 °C until further biochemical analysis.

2.3. Ethylene production

Ethylene production (pmol kg^{-1} s^{-1}) was measured as described by Giné-Bordonaba et al. (2017) with some modifications. Three replicates of 4 fruit each were placed in 2 L flasks sealed with a silicon septum for sampling the gas of the headspace after 2 h incubation in an acclimatized chamber at 20 °C. For the analysis of ethylene production, gas samples (1 mL) were taken using a syringe and injected into a gas chromatograph (GC; Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an alumina column F1 80/100 (2 m × 1/8 × 2.1, Tecknokroma, Barcelona, Spain) as previously described by Giné-Bordonaba et al. (2014).

2.4. Enzymes related to the ethylene metabolism

1-aminocyclopropane-1-carboxylic acid synthase enzyme (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase enzyme (ACO) were extracted from frozen flesh tissue and analysed as described by Lindo-García et al. (2019). The results were expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh weight basis.

1-aminocyclopropane-1-carboxylic acid (ACC) was extracted also from frozen flesh and analysed as described by Bulens et al. (2011) with some modifications. Briefly, 2 g of frozen tissue were homogenized with 4 mL of a 5 % (w/v) sulfosalicylic acid solution and vortexed until a homogenous mixture was obtained. The samples were then gently shaken for 30 min at 4 °C and centrifuged at 8,000 *g* for 10 min at 4 °C.

Subsequently, the supernatant was stored at -80 °C until analysis. The extract reading was performed mixing 1.4 mL of the ACC extract with 400 μL of 10 mmol L^{-1} $HgCl_2$ and 200 μL of a solution of NaOCl saturated with NaOH (2:1 v/v). After 4 min, a 1 mL headspace gas sample was injected into a gas chromatograph and the results expressed as μmol C_2H_4 kg^{-1} on a fresh weight basis.

2.5. Antioxidant capacity, hydrogen peroxide and malondialdehyde contents

Malondialdehyde (MDA), as an index of lipid peroxidation, was analysed as described by Martínez-Solano et al. (2005) using the thiobarbituric acid reactive substrates (TBARS) and the results expressed as nmol kg^{-1} s^{-1}. Antioxidant capacity was analysed using the Ferric Reducing Antioxidant Power (FRAP) assay as previously described by Giné-Bordonaba and Terry (2016). Results were expressed as g $FeCl_3$ kg^{-1} of fresh weight. H_2O_2 levels were determined as described by Giné-Bordonaba et al. (2017) using the Bioxytech H_2O_2-560 (OXIS International Inc., Portland, OR USA) colorimetric assay following the manufacturer's instructions. The content was expressed as mmol kg^{-1} of fresh weight.

2.6. Sugar and organic acid content

Malic acid and sugars (sucrose, glucose and fructose) were extracted from flesh frozen tissue as described by Giné-Bordonaba et al. (2017). Malic acid was extracted dissolving 2 g of frozen tissue in 5 mL of distillate water. The samples were slightly shaken for 10 min at room temperature and then centrifuged at 24,000 *g* for 7 min at 20 °C. The resulting supernatant was recovered and used for enzyme coupled spectrophotometric determination (L-malate dehydrogenase) of malic acid using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer instructions.

For sugars determination, 2 g of frozen flesh tissue were diluted in 5 mL of 62.5 % (v/v) aqueous methanol solvent and placed in a thermostatic bath at 55 °C for 15 min, mixing the solution with a vortex every 5 min to prevent layering. Then, the samples were centrifuged at 24,000 *g* for 15 min at 20 °C. The supernatants of each sample were recovered and used for enzyme coupled spectrophotometric determination of glucose and fructose (hexokinase/phosphoglucose isomerase) and sucrose (β-fructosidase) using commercial kits (BioSystems S.A., Barcelona, Spain) and following the manufacturer instructions.

2.7. Statistical Analysis

All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS Institute Inc. Mean comparisons between ripening conditions at specific days for each cultivar was done by Student's *t*- test ($p \leq 0.05$) using critical values of *t* for two-tailed tests. Least significant difference values (LSD; $p = 0.05$) for the interaction ripening condition*DACH were calculated for mean separation using critical values of *t* for two-tailed tests.

3. Results

3.1. Fruit quality changes during on- and off-tree ripening

Significant differences were observed when comparing the rate of firmness loss (softening) between on- and off-tree ripened fruit for both cultivars. 'Conference' pears (harvested at 62 N) experienced a sharp firmness loss from day 7 to day 21 (2.7 N d^{-1}) during off-tree ripening, reaching final firmness values of 5 N at day 21 (Fig. 1A). In contrast, the rate of firmness loss was much slower on-tree (0.2 N d^{-1}) and fruit never reached the optimal firmness for consumption (20-30 N; Torregrosa et al., 2019). In 'Flor d'Hivern' pears, firmness values were similar for both ripening scenarios until day 7 (*ca.* 48 N). Later, firmness values off-tree constantly decreased until reaching values of 26 N at day 28 whereas no firmness changes were observed on-tree (Fig. 1B).

Changes in I_{AD} values for both cultivars generally paralleled the changes observed in fruit firmness (Fig. 1; $r^2 = 0.94$ and 0.95 at $p \leq 0.05$ for 'Conference' and 'Flor d'Hivern', respectively). Briefly, in off-tree 'Conference' pears, I_{AD} value at harvest was 2.1 and then decreased by 5-fold at day 21 whereas relatively stable I_{AD} values (*ca.* 2) were observed in on-tree ripened fruit (Fig, 1C). A similar pattern was observed in 'Flor d'Hivern' pears but with lower values than those observed in 'Conference' pear (Fig. 1D).

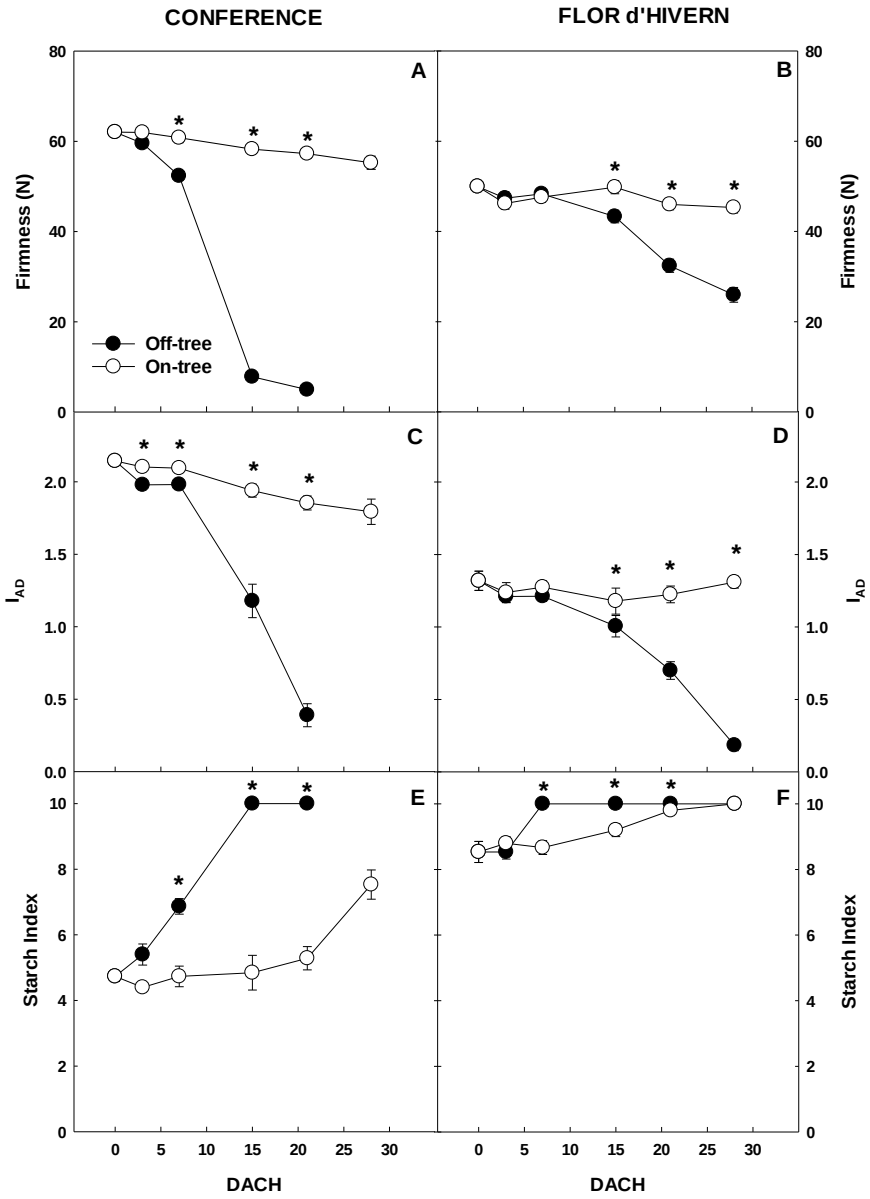

Figure 1. Changes in fruit firmness (A and B), I_{AD} (C and D) and starch index (E and F) during off-tree (●) and on-tree (○) ripening for 'Conference' (left) and 'Flor d'Hivern' (right) cultivars. DACH stands for Days After Commercial Harvest. Error bars represent the standard errors of the means (n=3). Stars indicate significant differences at $p \leq 0.05$. LSD values ($p \leq 0.05$) for the interaction ripening condition*DACH for figures A, B, C, D, E and F were: 3.02, 5.13, 0.21, 0.11, 0.95 and 0.40, respectively.

TSS content increased both on- and off-tree in 'Conference', yet a faster increase was observed in off-tree ripened fruit, reaching similar values (13.8 %) at day 21 and day 28, respectively (Table 1). The pattern observed in 'Flor d'Hivern' was however completely different. TSS content in off-tree ripened fruit was about 14 % and remained relatively unchanged along the storage period, while TSS content in on-tree ripened fruit decreased by 1.2-fold from day 7 to day 28 reaching final values of 11.6 % (Table 1).

Completely different patterns were also observed for the fruit acidity changes between cultivars. In 'Conference' pear, titratable acidity (TA) decreased both on- and off-tree but with values consistently higher in off-tree ripening (Table 1). TA values in 'Flor d'Hivern' remained similar until day 7 in both ripening scenarios. After this day, TA was maintained at 2.3 g malic L^{-1} in on-tree ripened fruit whereas it decreased by 1.3-fold in fruit ripened off-tree at 20 ºC (Table 1).

Table 1: Changes in total soluble solids (TSS; %) and titratable acidity (TA; g malic L^{-1}) during off- and on-tree ripening for 'Conference' and 'Flor d'Hivern' cultivars. Means ± standard error followed by the same letter at each day for each cultivar are not significant different at $p \leq 0.05$ (n=3). LSD values ($p \leq 0.05$) for the interaction ripening condition*days in 'Conference' cultivar were 0.73 and 0.27 for TSS and TA, respectively; and in 'Flor d'Hivern', 0.70 and 0.29, respectively.

TSS (%)				
	'Conference'		'Flor d'Hivern'	
Days	Off-tree	On-tree	Off-tree	On-tree
0	12.8 ± 0.09 a	12.8 ± 0.09 a	13.6 ± 0.12 a	13.6 ± 0.12 a
3	12.6 ± 0.13 a	12.2 ± 0.19 a	13.6 ± 0.03 a	13.7 ± 0.25 a
7	13.0 ± 0.23 a	12.0 ± 0.23 b	13.7 ± 0.27 a	13.6 ± 0.03 a
15	13.5 ± 0.12 a	12.2 ± 0.29 b	13.8 ± 0.18 a	12.9 ± 0.12 b
21	13.9 ± 0.30 a	12.9 ± 0.28 a	13.3 ± 0.09 a	12.7 ± 0.09 b
28		13.7 ± 0.42	13.8 ± 0.58 a	11.6 ± 0.26 b

TA (g malic L^{-1})				
	'Conference'		'Flor d'Hivern'	
Days	Off-tree	On-tree	Off-tree	On-tree
0	2.1 ± 0.11 a	2.1 ± 0.11 a	2.2 ± 0.04 a	2.2 ± 0.04 a
3	1.6 ± 0.12 a	1.7 ± 0.05 a	2.1 ± 0.05 a	2.2 ± 0.06 a
7	1.7 ± 0.14 a	1.6 ± 0.04 a	2.3 ± 0.05 a	2.3 ± 0.18 a
15	1.6 ± 0.12 a	1.2 ± 0.10 a	1.8 ± 0.11 b	2.5 ± 0.03 a
21	1.5 ± 0.05 a	1.3 ± 0.06 b	1.8 ± 0.15 b	2.3 ± 0.07 a
28		1.1 ± 0.05	1.8 ± 0.12 b	2.4 ± 0.10 a

Starch Index (SI) in 'Conference' pears ripened off-tree reached the maximum value (10) after 15 d at 20 ºC, whereas on-tree ripened fruit showed a constant SI value until day 21. Later, SI increased by 1.4-fold reaching a value of 7.5 at day 28 (Fig. 1E). On the other hand, SI of 'Flor d'Hivern' pears at harvest almost doubled the value observed in 'Conference' pears, and reached the maximum value off-tree at day 7, whereas on-tree fruit did not reach this maximum value until day 28 (Fig. 1F).

The different patterns observed in off- and on-tree ripened fruit in these quality parameters may help to define the optimum harvest date for the different pear cultivars investigated herein improving then their final quality and storability.

3.2. Ethylene production and ACC metabolism

In 'Conference' pear, both on- and off-tree ripened fruit showed a similar pattern of ethylene production until day 15. Later, ethylene production off-tree increased about 6-fold, reaching a value of 0.7 pmol kg^{-1} s^{-1} whereas ethylene production remained low on-tree until day 21 and increased later to 2 pmol kg^{-1} s^{-1} at day 28. Ethylene production in 'Flor d'Hivern' was very low (values ranging from 0.08 to 0.24 pmol kg^{-1} s^{-1}) and did not show any clear pattern when comparing on- and off-tree ripened fruit (Fig. 2).

ACS activity in off-tree ripened 'Conference' pear increased from day 7 by 2.3-fold to reach values of 0.07 nmol $kg^{-1}s^{-1}$ at day 21 whereas no changes were noticed in on-tree ripened fruit (stable values around 0.03 nmol kg^{-1} s^{-1} during the ripening process; Fig. 2).

However, in 'Flor d'Hivern' pears, no clear pattern was observed regarding ACS activity. (Fig. 2).

Significant differences in the ACC content were found between ripening conditions in 'Conference' pears. ACC levels in on-tree fruit were constant along all the ripening process, however, off-tree fruit showed a peak of ACC content at day 15 (0.25 μmol kg^{-1}; 14.5-fold higher than values observed from harvest to day 7) and ACC levels slightly declined thereafter until day 21. In 'Flor d'Hivern' pears, no clear peaks of ACC could be detected and ACC values were generally higher in on- than in off-tree ripened fruit (Fig. 2).

In contrast to the sound differences between cultivars detailed earlier, both cultivars showed a sharp increase of ACO activity in off-tree ripened fruit. ACO activity increased from day 3, reaching maximum values of 2 nmol kg^{-1} s^{-1} at day 15 and 1.5 nmol kg^{-1} s^{-1} at day 28 for 'Conference' and 'Flor d'Hivern', respectively (Fig. 2). On-tree ripened fruit maintained stable values during all the ripening process, although 'Conference' showed a slight increase from day 21 to day 28, paralleled to the slight increase observed in ethylene production (Fig. 2).

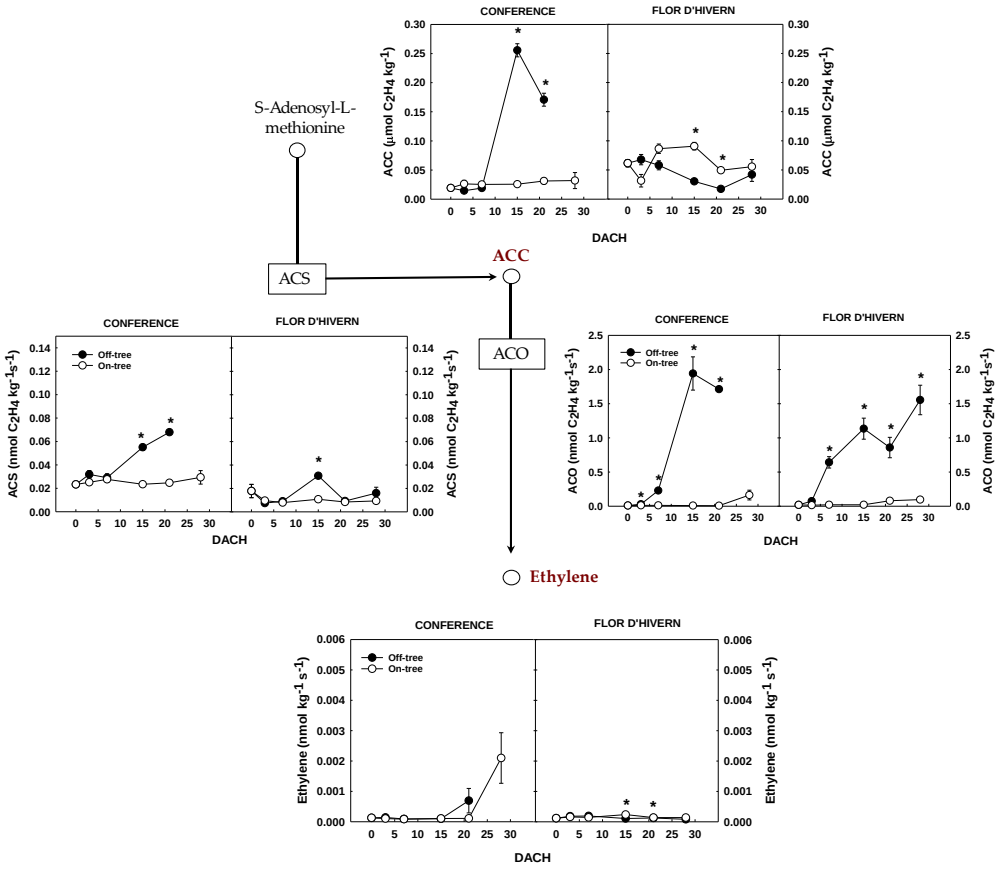

Figure 2. Ethylene metabolism scheme showing the ethylene production, ACC synthase activity, ACC content and ACC oxidase activity during off-tree (●) and on-tree (○) ripening for 'Conference' and 'Flor d'Hivern' cultivars. DACH stands for Days After Commercial Harvest. Error bars represent the standard errors of the means (n=3). Stars indicate significant differences at $p \leq 0.05$. LSD values ($p = 0.05$) for the interaction ripening condition*DACH for ACS, ACC, ACO and ethylene production were: 0.007, 0.02, 0.16 and 0.54, respectively for 'Conference' pear and 0.008, 0.02, 0.26 and 0.06, respectively, for 'Flor d'Hivern' pears.

3.3. Oxidative and peroxidative changes

MDA levels in 'Conference' pears ripened off-tree gradually increased from harvest until day 21. On-tree ripened fruit did not show significant variations along the ripening process, maintaining values of 4 nmol kg^{-1} s^{-1} (Fig. 3A). A similar trend was observed in on- and off-tree ripened 'Flor d'Hivern' pears that exhibited unchanged MDA values for both ripening conditions (Fig. 3B).

Likewise, no clear differences were observed in the fruit antioxidant capacity when comparing on- and off-tree ripened fruit for any of the two cultivars (Fig. 3C and D).

Changes in H_2O_2 content were significantly different between on- and off-tree ripening for both cultivars. In 'Conference' pears off-tree, H_2O_2 content sharply decreased after a transient increase at day 3 to reach values of 15 mmol kg^{-1} at day 15 and thereafter. In contrast, H_2O_2 content on-tree remained fairly unchanged until day 21 to decline thereafter (1.4-fold lower at day 28 than at day 21; Fig. 3E). On-tree ripened 'Flor d'Hivern' pears showed two transient peaks of H_2O_2 at days 3 and 21 whereas H_2O_2 levels off-tree steadily decreased along the ripening process showing values at day 28 *ca.* 2-fold lower than those observed at harvest (Fig. 3F).

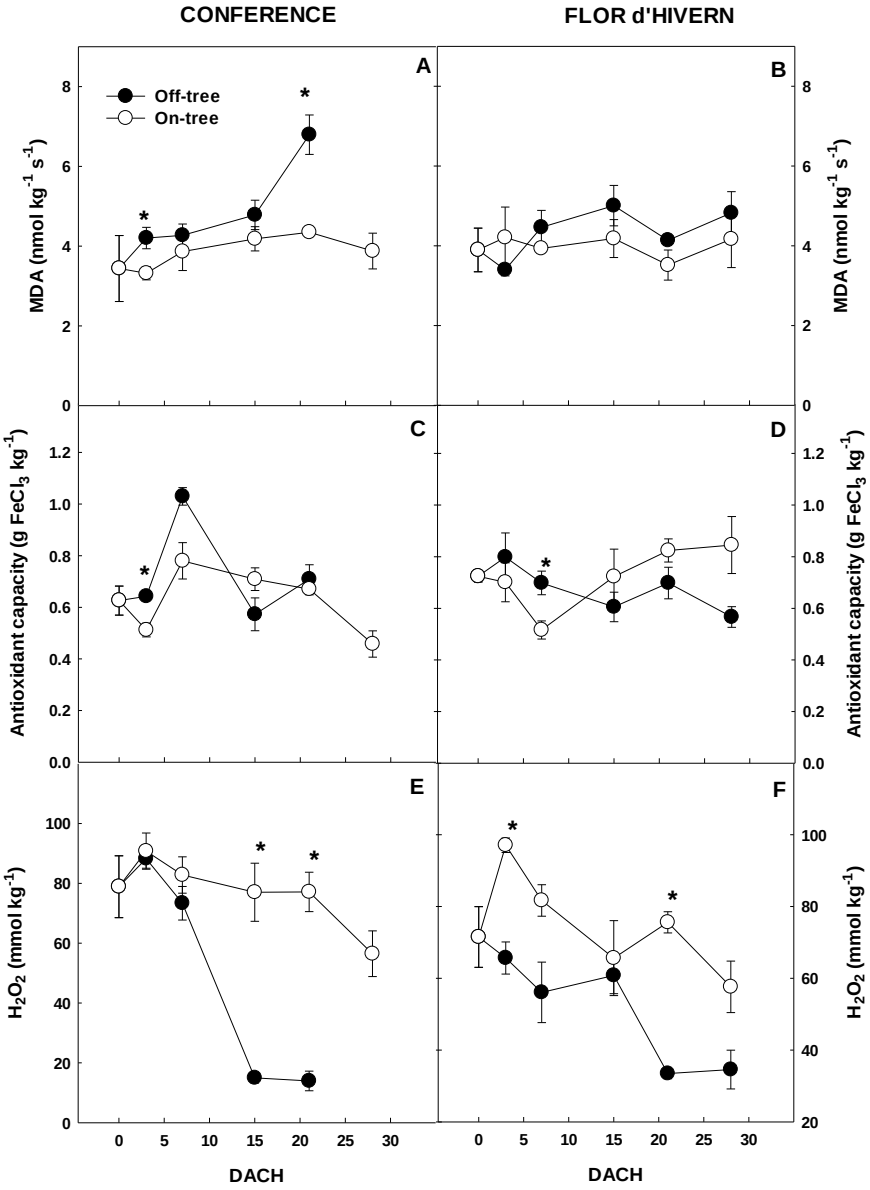

Figure 3. Changes in the concentration of malondialdehyde (A and B), antioxidant capacity (C and D) and changes in hydrogen peroxide (E and F) during off-tree (●) and on-tree (○) ripening for 'Conference' (left) and 'Flor d'Hivern' (right) cultivars. DACH stands for Days After Commercial Harvest. Error bars represent the standard errors of the means (n=3). Stars indicate significant differences at $p \leq 0.05$. LSD values ($p \leq 0.05$) for the interaction ripening condition*DACH for figures A, B, C, D, E and F were: 1.10, 1.42, 0.13, 0.20, 19.80 and 17.17, respectively.

3.4. Sugar and malic acid accumulation during off- and on-tree ripening

Slight differences in malic acid content were observed in 'Conference' pears when comparing on- and off-tree ripening. In this pear cultivar, malic acid content decreased in fruit ripened off-tree, and especially from day 7 onwards, but remained unchanged in fruit ripened on-tree (Fig. 4A). Similarly, malic acid content did not significantly differ between on- and off-tree ripened 'Flor d'Hivern' pears (Fig. 4B).

Main differences between on- and off-tree ripened fruit for 'Conference' pear were found in glucose content. Despite the decrease observed in both ripening conditions from harvest to day 3, glucose content increased later by 1.3-fold until day 21 off-tree but remained constant on-tree (Fig. 4C). However, glucose content in 'Flor d'Hivern' remained constant except a transient peak at day 3in on-tree ripened fruit (Fig. 4D).

In 'Conference' pears, off-tree ripened fruit showed a gradual decrease in fructose content from 65 g kg^{-1} at harvest to 53 g kg^{-1} at day 15 followed by an important increase (1.5-fold) thereafter. In contrast, fructose values slightly increased on-tree with a transient peak observed at day 7 (Fig. 4E). Fructose content in 'Flor d'Hivern' pears remained relatively unchanged along ripening (*ca.* 80 g kg^{-1}) regardless of the ripening conditions (Fig. 4F).

As for glucose, sucrose changes were different between on- and off-tree ripened fruit as well as between cultivars. Sucrose levels in 'Conference' pears doubled during the ripening process both on- and off-tree with values generally higher off-tree (Fig. 4G). In contrast, in 'Flor d'Hivern' pears, sucrose content decreased in attached fruit while the opposite pattern was observed in fruit detached to the tree (Fig. 4H).

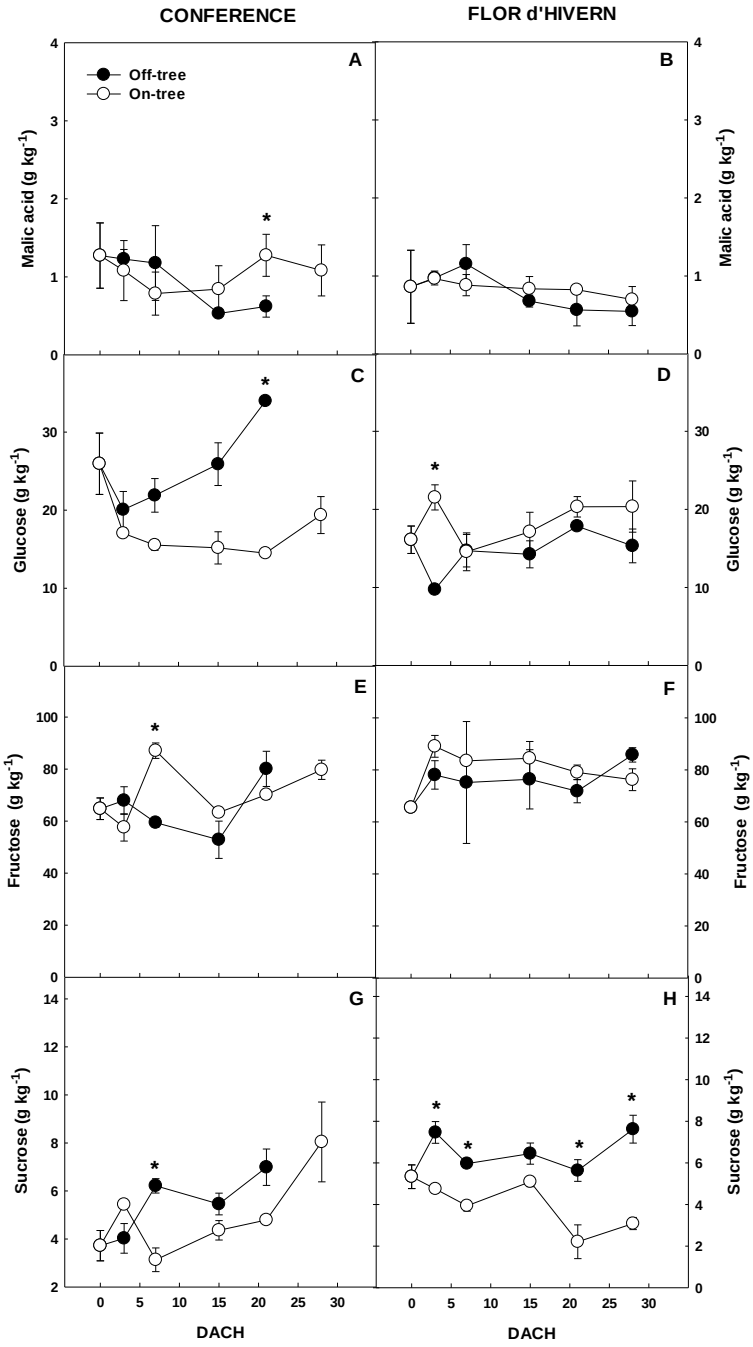

Figure 4. Changes in malic acid content (A and B), D-Glucose (C and D), D-fructose levels (E and F) and sucrose levels (G and H) during off-tree (●) and on-tree (○) ripening for 'Conference' (left) and 'Flor d'Hivern' (right) cultivars. DACH stands for Days After Commercial Harvest. Error bars represent the standard errors of the means (n=3). Stars indicate significant

differences at $p \leq 0.05$. LSD values ($p \leq 0.05$) for the interaction ripening condition*DACH for figures A, B, C, D, E, F, G and H were: 0.54, 0.32, 7.74, 6.23, 13.15, 20.50, 1.67 and 1.40, respectively.

4. Discussion

4.1. On- and off-tree pear ripening in pears is not strictly mediated by ethylene

In contrast to that described earlier for other summer pears cultivars (Lindo-García et al., 2019), on-tree ripening in 'Conference' pears was not accompanied by noticeable changes in fruit firmness or starch degradation. Generally, significant differences existed in most ripening related traits when comparing on- and off-tree ripening for this pear cultivar. This said, such differences were not strictly related to the fruit capacity to produce ethylene that remained at low levels in both conditions (Fig. 2). Firmness loss and degreening (Fig. 1A and Suppl. Fig. 2) observed in off-tree ripened fruit may be also related to an increase in ethylene sensitivity and to the low levels of ethylene produced by this cultivar. Accordingly, Johnston et al. (2009) reported that sensitivity to ethylene in apple increased as fruit ripen and that changes of some ripening traits may have different sensitivities to ethylene. Likewise, the increase in starch index in off-tree ripened 'Conference' pear may also be related to increased sensitivity to ethylene even though the relationship between starch degradation and ethylene is still controversial (Johnston et al., 2009; Singh et al., 2017).

The inhibition of ethylene production in 'Conference' pears during on-tree ripening was paralleled by a general inactivation of ACC metabolism (ACS and ACO). During off-tree ripening, the lack of ethylene production was not due to limited ACS activity as previously reported in 'Blanquilla' pears (Lindo-García et al., 2019) nor to a lack of ACC or inhibition of ACO activity. In this pear cultivar, temperature conditions when comparing on- and off-tree ripened fruit were fairly similar (Suppl. Fig. 1) thereby suggesting that differences in ethylene metabolism between on- and off-tree ripened fruit may not be linked to weather conditions. In this sense, other compounds such as hormones, sucrose or some molecules still unknown (Jia et al., 2013; Meyer et al., 2017) likely produced by the mother plant may be responsible for inhibiting the fruit ethylene production in 'Conference' pears (Lindo-García et al., 2020b; Nham et al., 2015).

A complex hormonal cross-talk leading to inhibited or enhanced ethylene production has been detailed in several species (Jiang et al., 2000; Trainotti et al., 2007; Zhang et

al., 2009). High gibberellin 1 content in 'Conference' pears when still attached to the tree may explain why this cultivar is not able to produce ethylene on-tree or immediately after harvest (Lindo-García et al., 2020b). This hypothesis is further supported by the fact that 'Conference' pears, like other pears, need a short chilling period to ripen properly (Hansen and Mellenthin, 1979; Villalobos-Acuña and Mitcham, 2008) and that low temperatures are indeed known to decrease the content of gibberellins in other plants (Pinthus et al., 1989; Reid et al., 1974).

During on-tree ripening, the very low ethylene production in 'Conference' may be also attributed to a restricted ACC metabolism since ACS and ACO activity as well as ACC levels remained low and unchanged. These findings are consistent with the theory of the 'tree factor' (Abeles et al., 1992) in which an ethylene inhibitor is exported from the leaves to the fruit via the phloem (Sfakiotakis and Dilley, 1973) limiting on-tree ripening. Such theory has been extensively investigated in avocado fruit (Liu et al., 2002; Pedreschi et al., 2014; Tingwa and Young, 1975), a climacteric fruit unable to ripen unless detached from the tree. As said, gibberellins as well as other hormones (such as jasmonic or salicylic acids) may account for the observed inhibition of ACC metabolism (Kondo et al., 2007; Lindo-García et al., 2020b; Zhang et al., 2003).

Similarly to that observed for 'Conference' pears, the observed changes in fruit softening or degreening (Fig. 1B and Suppl. Fig. 2) in detached 'Flor d'Hivern' fruit were neither explained by the fruit ethylene production capacity. This pear cultivar did not produce ethylene during either on- or off-tree ripening behaving like a non-climacteric fruit and showing an unusual ACC metabolism. In fact, even the low temperatures observed during on-tree ripening if compared to those of off-tree ripened fruit (Fig.5) were not sufficient to induce the ethylene production in this cultivar. Whether ethylene sensitivity is different when comparing on- and off-tree ripened fruit is still unknown and warrants further investigations.

Even though ethylene did not increase in detached fruit, ACO activity was clearly enhanced during off-tree ripening, reaching levels similar to those observed in 'Conference'. Since ACC is commonly conjugated to an inactive form, the malonyl ACC (MACC; (de Poel and Van Der Straeten, 2014)), inhibition of ethylene production and ACC deficiency during off-tree ripening in 'Flor d'Hivern' might be due to an increase in MACC. Our results for this specific pear cultivar suggest, for the first time, the existence of a non-climacteric-like cultivar among the *Pyrus communis*. Previous

studies carried out in other Pyrus spp. have already identified the existence of non-climacteric like cultivars (i.e. cv. 'Nijisseiki'; *Pyrus pyrifolia*) nor producing or responding to propylene treatments (Downs et al., 1991). This said, further works should determine the molecular regulation of ripening impairment in 'Flor d'Hivern' to further confirm this hypothesis.

4.2. An oxidative process may be responsible for triggering off-tree ripening in some pear cultivars

In a previous study, the initiation of fruit softening and ripening in 'Blanquilla' pears was associated to an oxidative stress leading to higher MDA content and preceding the climacteric rise (Lindo-García et al., 2019). Similar results were found in this work in 'Conference' pears in which softening and increase in lipid peroxidation (MDA content) clearly precede the initiation of ethylene production in off-tree ripened fruit. Although these changes were much more limited in 'Conference' than in 'Blanquilla' pears (Lindo-García et al., 2019), and to some extent the MDA content measured by the methodology described herein may be overestimated, our results indicate that oxidative processes may be key factors that trigger the ripening capability in summer pears.

Since the levels of H_2O_2 decreased during off-tree ripening (Fig. 3E), the oxidative processes leading to higher MDA content were not likely mediated by H_2O_2, nor accompanied by a decline in the fruit antioxidant capacity (Fig. 3C). These findings differ from previous studies carried out in tomato (Kumar et al., 2016) and cherry fruit (Giné-Bordonaba et al., 2017) in which H_2O_2 levels were reported to increase along the ripening process. Nonetheless, they are in accordance with those observed in 'Blanquilla' (Larrigaudière et al., 2004) and 'Conference' pears (Larrigaudière et al., 2001) during postharvest cold storage.

During on-tree ripening, and despite of the higher H_2O_2 levels if compared to off-tree ripening, MDA content in 'Conference' pears only increased slightly in accordance with the observed low softening rate. A similar behaviour was found for 'Flor d'Hivern' pears that exhibited constant levels in lipid peroxidation markers despite significant differences in H_2O_2 levels between these two ripening scenarios. Collectively, these results clearly indicate that pears, when attached on-tree, very effectively impaired the action of H_2O_2 and oxidative damage. This behaviour may explain the differences in ripening behaviour observed on-tree and is in accordance

with an idea that the 'tree factor' is not only associated with an inhibition of ACC metabolism but also with the endogenous capacity of pears to prevent oxidative damage on-tree. Further studies that may consider the putative roles of lipoxygenases and antioxidant enzymes, among others, are needed to better determine the real nature of the 'tree factor'.

4.3. The role of assimilates during the ripening process in summer vs. winter pears

With this in mind, we further analysed the putative role that photo-assimilates may play during on- and off-tree pear ripening. Among them, sucrose has been reported to act as an important signal molecule regulating fruit development and ripening in both climacteric and non-climacteric species (Jia et al., 2013). In 'Blanquilla' pear, the increase in sucrose content was concomitant with the increase in the fruit ethylene production and thereby the ability to ripen on-tree (Lindo-García et al., 2019). In this work, sucrose levels also increased in 'Conference' pears but remained unchanged in 'Flor d'Hivern' on-tree, being always at levels significantly lower than in 'Blanquilla' pears (i.e. maximum of about 8 g kg^{-1} in 'Conference' and 'Flor d'Hivern' vs. 20 g kg^{-1} in 'Blanquilla'; Lindo-García et al., 2019). In 'Blanquilla', the ethylene production was initiated only when sucrose levels were higher than 10 g kg^{-1}. Collectively these results suggest then that this value might be a threshold value that has to be reached to initiate ethylene production among different pear cultivars.

On the other hand, the unexpected decrease from day 7 (Fig. 4H) in sucrose content during on-tree ripening of 'Flor d'Hivern' pears was in accordance with the TSS changes (Table 1). Such a decrease may be triggered by the lower field temperatures likely causing sucrose breakdown as commonly observed during cold storage of apples and pears (Drake and Eisele, 1999; Itai and Tanahashi, 2008).

In addition to sucrose, other sugars as well as malic acid play an essential role in many processes during fruit development and ripening (Ciereszko, 2018; Fernie and Martinoia, 2009). The decrease in malic acid along off-tree ripening for both cultivars may be explained by its function as a respiratory substrate (Famiani et al., 2014) as previously described in apple (Liu et al., 2016) and 'Blanquilla' pears (Lindo-García et al., 2019). On another hand, the sharp increase in glucose content observed in 'Conference' off-tree but not in 'Flor d'Hivern' pears, is likely the reflection of the ripening-related events resulting from the degradation of complex sugars (i.e. starch)

to glucose. In this sense, further studies investigating the influence of exogenous sugar applications on pear ripening, both on- and off-tree, are warrant.

5. Conclusions

The results from this study provide new information about the complex ripening process of fruit attached and detached to the tree among different European pears. They may be useful to understand the ripening physiology of pear or even to decide the optimum harvest date among the studied cultivars. Unlike other summer pear cultivars, our results showed that 'Conference' pear was not able to completely ripen (soften) on-tree. In this cultivar the ripening impairment observed on-tree was a consequence of low ACC metabolism. In contrast, during off-tree ripening, softening and colour changes seemed to be triggered by an oxidative process and later by an enhanced ACC metabolism, yet not being accompanied by higher fruit ethylene production. On the other hand, the winter pear 'Flor d'Hivern' owns an unusual ACC metabolism and high resistance to oxidative damage, behaving to some extent like a non-climacteric fruit. This said this pear cultivar also experienced some softening and degreening during off-tree ripening which are triggered so far by unknown causes.

6. Author's contribution

JGB, CL and VLG conceived and designed the experiment. VLG and ED performed all field and storage samplings including quality measurements and sample preparation for biochemical analysis. VLG, GE and MLL performed the analysis of ethylene and ethylene-related enzymes or precursors. VLG, CL and JGB wrote the manuscript and all remaining authors contributed in improving and revising the final version.

7. Declaration of Competing Interest

All authors declare no conflict of interest.

8. Acknowledgments

This work has been supported by the CERCA Programme/ Generalitat de Catalunya and by the Ministerio de Economía y Competitividad / MINECO (grant AGL2017-87923-R). Thanks are also given to the Department d'Empresa i Coneixement de la Generalitat de Catalunya and the European Social Fund 'Investing

in your future' for the predoctoral fellowship to VLG (2019FI-B2-00218). We are grateful to Dolors Ubach for her technical support.

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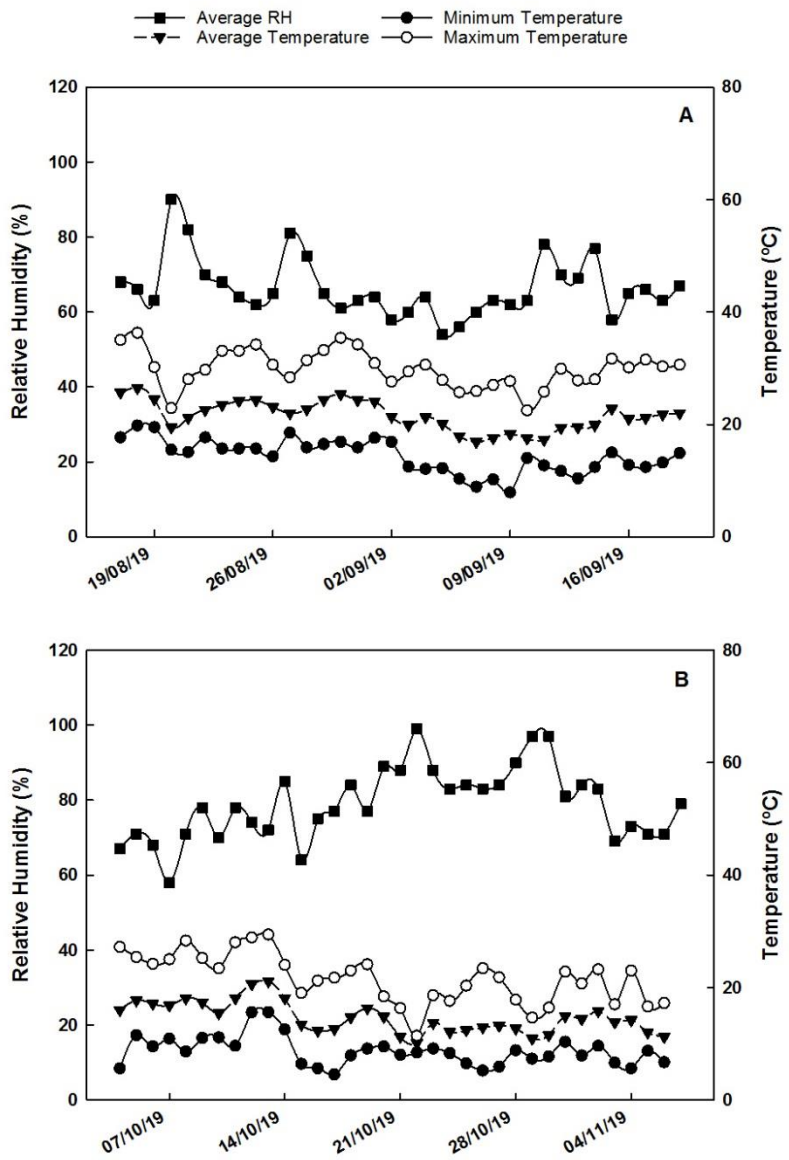
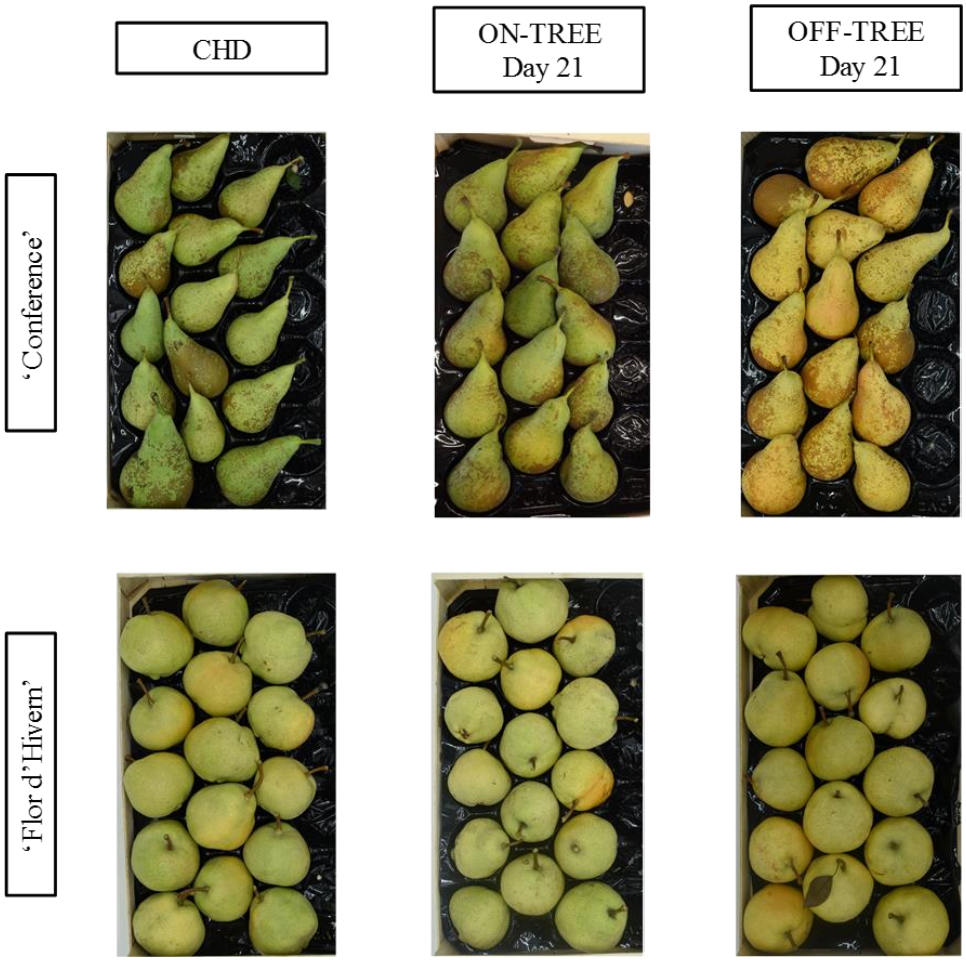
Supplementary data



Supplementary Figure 1: Temperature and relative humidity during the period between samplings for on-tree ripened 'Conference' (A) and 'Flor d'Hivern' (B) pears. Off-tree ripened fruit were stored at 20 ± 0.5 °C and 90 % RH.



Supplementary Figure 2: Degreening observed along the ripening process for 'Conference' and 'Flor d'Hivern' pears. Images were taken at harvest and after 21 days of on- and off-tree ripening. CHD means 'Commercial Harvest Date'.

CAPÍTULO 4

The relationship between ethylene- and oxidative-related markers at harvest with the susceptibility of pears to develop superficial scald

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*Postharvest Biology and Technology (2020), h*ttps://doi.org/10.1016/j.postharvbio.2020.111135

Abstract

To better understand the specific biochemical pathways involved in superficial scald susceptibility, changes in ethylene biosynthesis, antioxidant, oxidative related processes and sugar metabolism were investigated for two scald sensitive pear cultivars ('Blanquilla' and 'Flor d'Hivern') with distinct postharvest ripening patterns at different harvest dates. Both cultivars developed symptoms of scald after 4 months of storage at -0.5 °C, but the biochemical basis underlying susceptibility were different. In the summer pear 'Blanquilla', capable of ripening even on the tree, scald susceptibility was higher in fruit of advanced maturity and was associated with the action of ethylene on triggering the expression of *PcAFS1* gene. In this cultivar, the levels of ACC, ACS enzyme activity and *PcAFS1* at harvest were strongly correlated to scald incidence. In contrast, in the winter pear, 'Flor d'Hivern', with little or no ethylene-production capacity even after cold storage, scald symptoms were already visible when fruit were removed from cold storage, regardless of the fruit maturity. In this pear cultivar, scald symptoms were not dependent on ethylene, but rather associated with higher lipoxygenase (LOX) activity at harvest, an enzyme often associated with responses to chilling injury, and lower content of sorbitol, a compound that may act as cryoprotectant preventing cell damage during cold storage.

Keywords: ACC metabolism, chilling injury, peroxidative damage, sorbitol, superficial scald.

1. Introduction

Superficial scald is considered one of the major cold-storage disorders affecting pome fruit and leading to substantial economic losses worldwide (Lurie and Watkins, 2012). The disorder is generally induced during cold storage and the symptoms, which manifests as brown or dark patches on the fruit skin, generally appearing after cold storage (Hui et al., 2016). Senescent scald is another storage disorder described in pear fruit, which manifests with similar symptoms on the fruit skin yet quickly progressing to the flesh when the fruit is left at room temperature (Whitaker et al., 2009; Zoffoli et al., 1998).

Research on superficial scald of pome fruit is abundant (Lurie and Watkins, 2012; Larrigaudière et al., 2016; Whitaker, 2013; Zhou et al., 2017) and, for decades, it has been assumed that the physiological or biochemical processes that determine scald susceptibility in apples and pears were equivalent. For instance, it was generally recognized that ethylene plays a determining role in scald development through the up-regulation of α-farnesene synthase (AFS) gene expression (Gapper et al., 2006; Pechous et al., 2005). Although this regulatory mechanism has been confirmed in different apple cultivars (Lurie et al., 2005), controversial information has been described for pears. For instance, 'Beurré d'Anjou' pears, although producing very low ethylene levels compared to 'Packham's Triumph', tend to accumulate similar α-farnesene levels during cold storage (Larrigaudière et al., 2016). A similar lack of relationship between ethylene and α-farnesene was also found in 'Beurré d'Anjou' pears picked at different harvest dates (Calvo et al., 2015), but also in apple lines suppressed for ACC metabolism (Pesis et al., 2009) or apple selections resistant and susceptible to superficial scald (Rao et al., 1998). Indeed, an ethylene-independent regulation of superficial scald has been recently described for apples (Busatto et al., 2018; Karagiannis et al., 2018).

The role that α-farnesene and its oxidation products (CTols) play in the development of scald has also been questioned. Given that scald is considered the result of an oxidative damage (Lurie and Watkins, 2012), such disorder was initially attributed to the oxidative action of CTols (Huelin and Murray, 1966). Indeed, numerous studies have shown that higher accumulation of this oxidation products was accompanied by greater scald incidence in apples (Giné Bordonaba et al., 2013; Moggia et al., 2010) and pears (Hui et al., 2016; Whitaker et al., 2009). However, studies describe inconsistencies in the α-farnesene hypothesis (Knee and Hatfield, 1981; Rao et al.,

1998; Whitaker et al., 2000). It has been suggested that α-farnesene oxidation is a mere consequence of free radical reactions occurring during chilling stress and that α-farnesene is not required for the induction of scald but rather in aggravating the symptoms in fruit already compromised by oxidative stress (Rao et al., 1998; Rupasinghe et al., 2000). Busatto et al. (2014) found that scald in apples was related to a specific accumulation of chlorogenic acid and to its further oxidation in brown pigments by polyphenol oxidase (PPO) enzyme and that CTols were acting as signalling molecules. Busatto et al. (2018) hypothesized that scald resistance in 1-MCP-treated fruit was linked to higher levels of unsaturated long chain fatty acids as well as sorbitol, a compound stabilising the cell membranes and likely acting as cryoprotectant, thereby providing the fruit with greater tolerance to chilling injury damage.

It has also been suggested that scald mainly arises from an imbalance between the fruit capacity to produce and/or regenerate antioxidants and hence scavenge reactive oxygen species produced during cold stress (Guerra et al., 2012; Ju and Bramlage, 1999; Silva et al., 2010). In this sense, interesting associations were found with increased levels of p-coumaryl fatty-acid esters (Du and Bramlage, 1993; Whitaker, 1998), α-tocopherol (Rudell et al., 2009) and ascorbate levels in fruit skin (Larrigaudière et al., 2016; Wang et al., 2018), and reduced scald incidence in different pome fruit cultivars. Nevertheless, attempts to link scald development with changes in the fruit enzymatic antioxidant potential have been so far inconclusive.

Most efforts dedicated to find predictive markers for scald susceptibility in apple and pears have been carried out by analysing changes in quality-related or biochemical markers during the first months of cold storage (Giné Bordonaba et al., 2013). Interesting prediction techniques based on CTol kinetics (Giné Bordonaba et al., 2013), chlorophyll fluorescence and colorimetric parameters (Guerra et al., 2012) as well as the emission of specific volatile compounds (Farneti et al., 2015) have been proposed in different cultivars. Few studies have attempted to discriminate the fruit susceptibility to scald based on performing a biochemical characterisation at harvest (Barden and Bramlage, 1994; Emongor et al., 1994).

Accordingly, the aim of this study was to explore the relationships between the concentration of specific compounds in pear fruit from different cultivars, harvest dates and superficial scald susceptibility. Emphasis was given to investigate biochemical compounds or the expression of genes involved in ethylene, antioxidant,

and α-farnesene metabolisms but also in other metabolic pathways recently hypothesized to play an important role on scald development such as sorbitol and PPO.

2. Material and methods

2.1. Plant materials and experimental design

About 156 fruit of 'Blanquilla' and 156 fruit (52 fruit per replicate) of 'Flor d'Hivern' pears (*Pyrus communis* L.) were harvested from 3 replicates of 5 trees per replicate on a commercial orchard near Lleida (Catalonia, Spain). Fruit were harvested at four different dates: about 5 d before the initial commercial harvest date (CHD-5), at the initial commercial harvest date (CHDi), the final commercial harvest date (CHDf; 3 days after CHDi) and 4 d after the final commercial harvest date (CHD+4). Immediately after harvest, fruit were transferred to the laboratory for biochemical analysis. Sixty fruit per harvest and cultivar (3 replicates of 20 fruit each) were stored in semi-commercial chambers routinely aired at -0.5 °C and 90 % of relative humidity for 4 months to evaluate storage disorders and 6 fruit (3 replicates of 2 fruit each) were also stored at the same conditions to evaluate the ethylene production after 30 d of cold storage. To analyse the quality parameters at harvest, 60 fruit (4 pseudo-replicates per each of three replicates of 5 fruit each) were used. The ethylene production capacity was evaluated on 6 fruit (3 replicates of 2 fruit each) whereas 9 fruit (3 replicates of 3 fruit each) were used to analyse the α-farnesene and conjugated trienols content. In parallel, 15 fruit (3 replicates of 5 fruit each) were used for biochemical measurements. To do so, 30 g of flesh from the equatorial zone of the fruit and 30 g of peel tissue were removed and immediately frozen in liquid nitrogen before being kept at -80 °C.

2.2. Quality evaluations at harvest

Fruit firmness was evaluated with a hand-held penetrometer (T.R. Turoni srl., Italy) equipped with and 8 mm probe as described by Chiriboga et al. (2011). Total soluble solids (TSS; %) were measured on pear juice using a digital hand-held refractometer (PAL-1, Atago, Tokyo, Japan) whereas titratable acidity (TA) concentrations were measured on the same juice by titration using 0.1 N NaOH and the results expressed as g malic acid L^{-1}. The starch index was evaluated using 5 fruit per replicate as described by Lindo-García et al. (2019).

2.3. Ethylene production

Ethylene production (nmol kg^{-1} s^{-1}) was measured as described by Giné-Bordonaba et al. (2014) with some modifications. Briefly, fruit were placed in 1.5 L flasks continuously ventilated with humidified air at a flow rate of 1.5 L h^{-1}. Gas samples (1 mL) were taken of effluent air using a 1 mL syringe and injected into a gas chromatograph (CG; Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an Alumina column F1 80/100 (2 m x 1/8 x 2.1, Tecknokroma, Barcelona, Spain). The oven temperature was 140 °C while the injector and detector were kept at 180 and 280 °C, respectively.

2.4. Enzymes related to the ethylene metabolism

1-Aminocyclopropane-1-carboxylic acid synthase (ACS) and 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) enzymes were extracted from frozen flesh tissue and analysed as described by Lindo-García et al. (2019). Enzyme activity was expressed as nmol C_2H_4 kg^{-1} s^{-1} on a fresh weight basis. 1-Aminocyclopropane-1-carboxylic acid (ACC) and malonyl-ACC (MACC) were extracted as described by Bulens et al. (2011) with some modifications. Briefly, 2 g of frozen flesh tissue were homogenized with 4 mL of a 5 % (w/v) sulfosalicylic acid solution. The samples were gently shaken for 30 min at 4 °C and then were centrifuged at 8,000 *g* for 10 min at 4 °C. Subsequently, the supernatant was stored at -80 °C until analysis. An aliquot of 0.5 mL of the supernatant was hydrolysed by adding 0.2 mL of 6 M HCl, vortex 5 s and incubated at 99 °C for 3 hours. Thereafter, tubes were removed from the dry bath and allow them to cool down for 15 min at room temperature. Then the reaction was neutralized by adding 0.2 mL of 6 M NaOH and vortex 5 s. The samples were centrifuged for 15 min at 20,000 *g* at 20 °C and the supernatant stored at -20 °C until analysis. ACC and MACC extracts reading was analysed as described by Bulens et al. (2011) and the results expressed as μmol C_2H_4 kg^{-1} on a fresh weight basis.

2.5. Determination of *α*-farnesene and conjugated trienols (CTols)

α-farnesene and CTols were analysed as described by Larrigaudière et al. (2019) with some modifications. A strip of the peel was removed from the equatorial zone of each fruit and 6 discs (10 mm diameter) prepared using a cork borer. The discs were immersed in 5 mL of HPLC grade hexane for 10 min with constant stirring and then the solution was filtered and mixed with hexane until a final volume of 5 mL.

Measurements were performed calibrating first the equipment with HPLC grade hexane. Absorbance at 232 nm (α-farnesene) and 281-290 nm (conjugated trienols) was recorded using a UV-spectrophotometer (1001 Plus, Milton Roy, USA). Concentrations of α-farnesene and conjugated trienols were calculated using the molar extinction coefficients E_{232nm}= 27,700 for α-farnesene and $E_{281-290nm}$ = 25,000 for conjugated trienols and the results expressed as μmol kg^{-1} on a fresh weight basis.

2.6. Fruit antioxidant capacity and antioxidant enzymes

Antioxidant capacity was analysed from peel tissue using the Ferric Reducing Antioxidant Power (FRAP) assay as previously described by Giné-Bordonaba and Terry (2016). Results were expressed as g Fe^{3+} kg^{-1} of fresh weight. Ascorbic acid was extracted and analysed as described by Rassam and Laing (2005) with some modifications. Briefly, 3 g of peel tissue was homogenized with 5 mL of metaphosphoric acid suspension (3 % MPA, 8 % acetic acid) and then centrifuged at 20,600 *g* for 22 min at 4 °C. The supernatant was filtered using a 0.22 μm Millipore filter. Total ascorbic acid were prepared mixing 950 μL of extract with 50 μL of 40 mM Tris [2-carboxiethyl] phosphine hydrochloride (TCEP·HCl) during 3 h at room temperature. Levels of total ascorbic acid were determined by injection of 10 μL of sample on an Agilent 1260 Infinity II liquid chromatograph UHPL measuring at 254 nm. Separation was carried out on a Poroshell 120 EC-C18 (3 x 100 mm, particle size 2.7 μm, Agilent) at a flow-rate of 0.125 ml min^{-1} using 10 % of methanol (v/v) as mobile phase.

Total peroxidase (POX, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.14.18.1) were extracted mixing 5 g of peel tissue with 10 mL of phosphate buffer (0.1M pH 6) with 0.5 mM cysteine and 5 % (w/v) PVPP. The extract was filtered through two layers of Miracloth and centrifuged at 20,000 *g* for 15 min at 4 °C. A 2.5 mL sample of the supernatant was loaded into a Sephadex G-25 column (PD 10; Pharmacia, Madrid, Spain) that had previously been equilibrated with 10 mL phosphate buffer pH 6 and the enzyme was eluted with 3.5 mL of the same buffer. The activity was analysed as described by Giné-Bordonaba et al. (2017). Lipoxygenase (LOX, EC 1.13.11.12) extraction and analysis were carried out accordingly to Larrigaudière et al. (2001) with some modifications. Peel frozen tissue (5 g) was mix with 15 mL of extraction solution containing 0.1 M phosphate buffer pH 7.5, 2 mM DTT, 1 mM EDTA, 0.1 % (v/v) Triton X-100 and 1 % (w/v) PVPP. The homogenized was filtered through two layers of Miracloth and centrifuged at 25,000 *g* for 15 min at 4 °C. The enzyme

activity was measured spectrophotometrically at 234 nm using a solution containing 0.1 M phosphate buffer pH 8, 8.6 mM linoleic acid, 0.25 % (v/v) tween-20 and 10 mM NaOH.

For the extraction of catalase (CAT, EC 1.11.1.6), 5 g of peel frozen tissue were homogenized with 15 mL 0.1 M phosphate buffer pH 7.8, 2 mM DTT, 0.5 mM EDTA and 1.25 mM polyethylene glycol. Homogenates were filtered through two layers Miracloth and centrifuged at 20,000 *g* for 15 min at 4 °C. Samples were loaded into a Sephadex G-25 column and the activity measured as described by Giné-Bordonaba et al. (2017). Enzyme activity was expressed in Unit of Activity (UA) per milligram of protein, with one UA representing the quantity of enzyme responsible for a change in 1 absorbance unit per minute.

2.7. Fructose and sorbitol content

Fructose and sorbitol were extracted from frozen flesh tissue as described by Giné-Bordonaba et al. (2017). The supernatants were recovered and used for enzyme coupled spectrophotometric determination of glucose and fructose (hexokinase/phosphoglucose isomerase), sucrose (β-fructosidase) and sorbitol (sorbitol dehydrogenase) using commercial kits (BioSystems S.A., Barcelona, Spain and Megazyme, Co. Wicklow, Ireland, respectively) and following the manufacturer's instructions.

2.8. Determination of superficial scald incidence

Scald incidence was estimated visually after 4 months of cold storage and after 7 d of shelf life (20 °C) for each harvest as described by Larrigaudière et al. (2019), and calculated as a percentage of the total number of fruit.

2.9. RNA extraction and gene expression analysis

Total RNA was extracted from peel tissue using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St Louis, MO, USA). RNA quantity was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Scientific). Gene expression analysis was performed as described by Baró-Montel et al. (2019) using KAPA SYBR® Fast qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, USA) as polymerase master mix. Oligonucleotides used for RT-qPCR analysis were designed as described by Busatto et al. (2019). *Md8283* was used as

independent reference gene in all the experiment (Busatto et al., 2019, 2018). Results were expressed as Mean Normalized Expression (MNE) and calculated using the method described by Muller et al. (2002).

2.10. Statistical Analysis

All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS Institute. Comparisons between harvest time for each variety were done by Tukey's test at a significant level of $p \leq 0.05$. Least significant difference values (LSD; $p < 0.05$) were calculated for mean separation using critical values of t for two-tailed tests.

3. Results

3.1. Influence of the fruit maturity at harvest on disorder incidence

The quality parameters determined at harvest are presented in Table 1. Flesh firmness decreased progressively from 69.6 N at CHD-5 to 53.5 N at CHD+4 in 'Blanquilla' pears (-1.79 N d^{-1} during on-tree ripening). In contrast, no significant differences were observed in 'Flor d'Hivern' pears between harvest dates, which maintained the same firmness values during on-tree ripening (50 N). Only slight differences in TSS values were found for the two cultivars. However, TSS values were always higher in 'Flor d'Hivern' pears than in 'Blanquilla' pears, especially at CHD+4 (Table 1). The TA slightly decreased in 'Blanquilla' pears whereas 'Flor d'Hivern' values remained stable. The SI increased as 'Blanquilla' pears ripened on-tree, and no clear pattern was observed for 'Flor d'Hivern' pears, yet SI values for this cultivar were generally higher than those observed in 'Blanquilla' pears (Table 1).

Table 1: Flesh firmness, total soluble solids (TSS), titratable acidity (TA) and starch index (SI) for 'Blanquilla' and 'Flor d'Hivern' at different harvest date (CHD stands for Commercial Harvest Date). Means ± standard error followed by the same letter are not significant different at $p \leq 0.05$ (n=3) for each cultivar. LSD values ($p < 0.05$) for the interaction cultivar*harvest date for firmness, TSS, TA and SI were 2.52, 0.48, 0.38 and 1.12, respectively.

Cultivar	Harvest	Firmness (N)	TSS (%)	TA (g malic L^{-1})	SI
Blanquilla	CHD-5	69.6 ± 0.92 a	12.9 ± 0.08 c	3.6 ± 0.08 a	3.3 ± 0.21 c
	Initial CHD	62.3 ± 0.72 b	13.3 ± 0.06 bc	3.3 ± 0.07 ab	5.0 ± 0.02 b
	Final CHD	57.8 ± 0.61 c	13.8 ± 0.04 a	3.2 ± 0.07 ab	6.2 ± 0.20 a
	CHD+4	53.4 ± 0.64 d	13.5 ± 0.07 ab	3.0 ± 0.09 b	6.6 ± 0.13 a
Flor d'Hivern	CHD-5	49.8 ± 0.87 a	13.7 ± 0.15 b	2.9 ± 0.09 a	6.8 ± 0.50 ab
	Initial CHD	47.8 ± 0.80 a	13.9 ± 0.15 b	3.2 ± 0.10 a	6.6 ± 0.48 b
	Final CHD	49.2 ± 0.59 a	13.7 ± 0.19 b	3.0 ± 0.11 a	7.8 ± 0.29 ab
	CHD+4	51.5 ± 0.78 a	14.8 ± 0.09 a	3.3 ± 0.11 a	8.4 ± 0.29 a

After 4 months of cold storage, only minimal differences in scald incidence were found among harvest dates for 'Blanquilla' pear that exhibited 0 to 6 % of the disorder incidence (Fig.1A). However, after 7 d of shelf life, superficial scald incidence sharply increased, observing 33 % , 71 %, 78 % and 98 % of incidence for CHD-5, CHDin, CHDfin and CHD+4 respectively (Fig. 1A). Scald incidence in 'Flor d'Hivern' pears was very high upon removal from cold storage (ranging from 70 to 75 %), and reached nearly 100 % after 7 d of shelf life regardless of the fruit maturity at harvest (Fig. 1B).

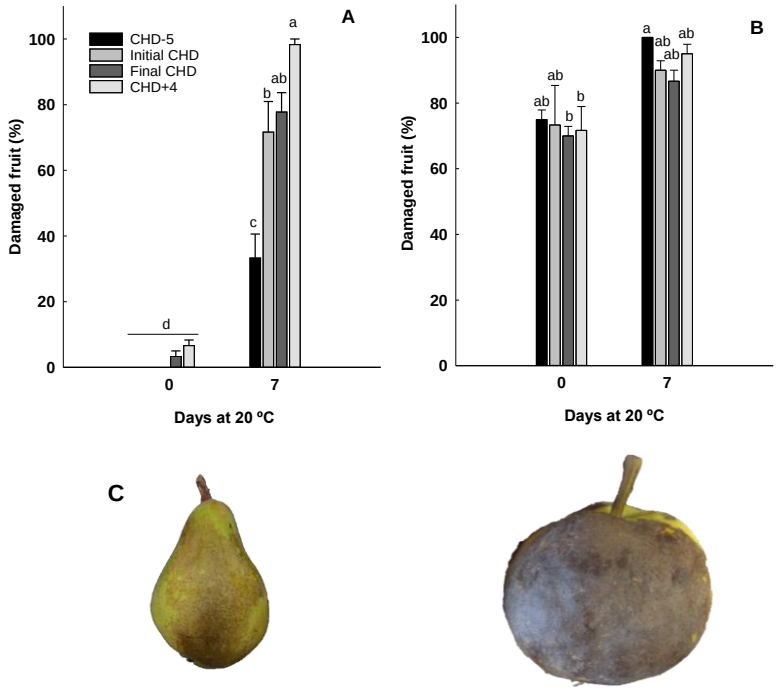

Figure 1: Scald incidence (%) in 'Blanquilla' (A) and 'Flor d'Hivern' (B) cultivars after 4 months of cold storage and 7 d of shelf life at 20 °C. Error bars represent the standard error of the mean (n=3). Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$. (C) Visual appearance of scald-like disorders in 'Blanquilla' (left) and 'Flor d'Hivern' (right) pears after 4 months of cold storage (-0.5 °C) plus 7 days of shelf-life (20 °C). CHD stands for Commercial Harvest Date.

3.2. Changes in ethylene metabolism

The ethylene production after harvest was notably different between cultivars. 'Blanquilla' pears produced ethylene immediately after harvest and the days needed to initiate ethylene production and reach the climacteric peak were reduced as the harvest date progressed. Fruit harvested at CHD-5 reached the ethylene peak (*ca.* 0.10 nmol kg^{-1} s^{-1}) after 20 d at 20 °C, whereas those harvested at CHD+4 need only 12 d at 20 °C (Fig. 2A). After 30 d of cold storage, 'Blanquilla' pears reached the ethylene peak at day 5, showing a maximum ethylene production of 0.10 nmol kg^{-1} s^{-1} (Fig. 2A, insert). In this pear variety, a short cold period did not lead to higher ethylene production values but rather to a faster initiation of the climacteric rise. Conversely, 'Flor d'Hivern' pears did not produce ethylene at harvest (maximum

ethylene production of *ca.* 0.014 nmol kg^{-1} s^{-1}; Fig. 2B) or even after a 30 days cold stress (max. 0.0062 nmol kg^{-1} s^{-1}; Fig.2B insert) regardless of the harvest date.

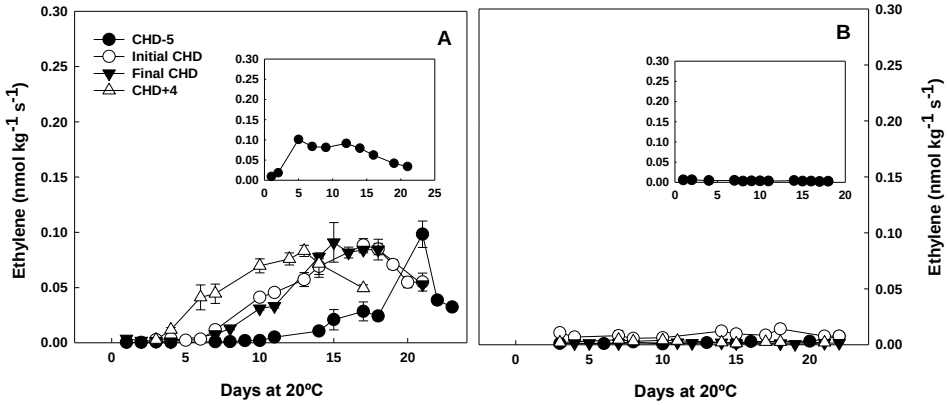

Figure 2: Ethylene production in 'Blanquilla' (A) and 'Flor d'Hivern' (B) cultivars at the different harvest dates. Error bars represent the standard error of the mean (n=3). Inserts describe the ethylene production at 20 °C after 30 d of cold storage of fruit harvested at final CHD. CHD stands for Commercial Harvest Date.

Differences in ethylene production rates between cultivars were related to differences in ACC metabolism. In 'Blanquilla' pears, ACC content increased by 15-fold from the earliest to the latest harvest date, reaching values of 6 μmol kg^{-1} at CHD+4. In contrast, in 'Flor d'Hivern' pears, ACC remained at very low levels of about 0.08 μmol kg^{-1} for all harvest dates (Fig. 3A), suggesting that the inability of this pear cultivar to produce ethylene was likely related to a reduced substrate availability. Regarding MACC content, no differences were observed between cultivars and harvest dates (with average values of 2 μmol kg^{-1}; Fig. 3B).

ACS activity in 'Blanquilla' pears increased from 0.01 nmol kg^{-1} s^{-1} at CHD-5 to 0.04 nmol kg^{-1} s^{-1} at CHD+4 hence supporting the results obtained for ACC. In contrast, ACS activity in 'Flor d'Hivern' pears remained constant (*ca.* 0.01 nmol kg^{-1} s^{-1}) at all the harvest dates (Fig. 3C). It is of interest to note that ACO activity showed an opposite pattern when comparing the cultivars. In 'Blanquilla' pears, ACO activity remained stable whereas in 'Flor d'Hivern' the enzyme activity increased by 4-fold, from the earliest (0.009 nmol kg^{-1} s^{-1}) to the latest harvest (0.04 nmol kg^{-1} s^{-1}; Fig. 3D). Although higher *PcACS1* gene expression was observed in 'Flor d'Hivern' pears compared with 'Blanquilla' pears (Fig. 3E), no significant

differences among harvest dates were observed for the cultivars. Furthermore, a clear up-regulation of *PcACO1* was observed in 'Flor d'Hivern' pears but expression of the gene was not affected by harvest date for either cultivar (Fig. 3F).

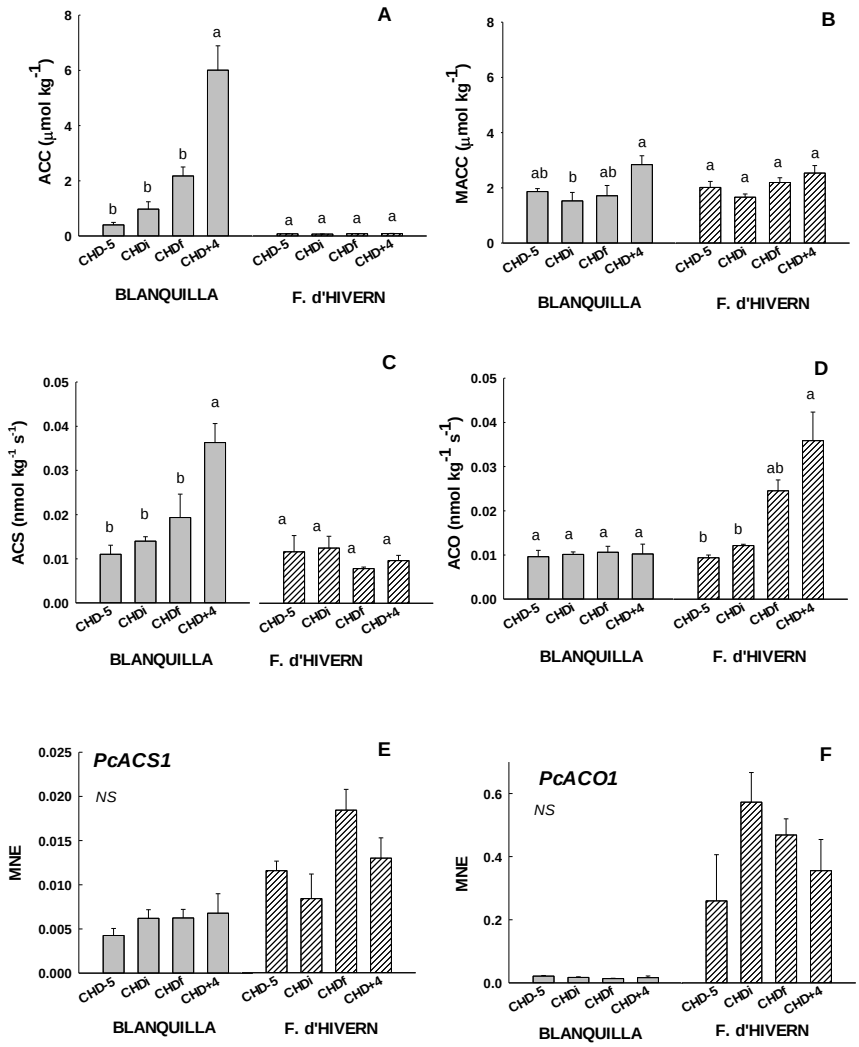

Figure 3: Content of ACC (A), MACC (B), and ACC synthase activity (C), ACC oxidase activity (D), *PcACS1* gene mean normalised expression (MNE; E) and *PcACO1* gene mean normalised expression (F) for 'Blanquilla' (grey bars) and 'Flor d'Hivern' (striped bars) pears. Error bars represent the standard error of the mean (n=3). LSD values ($p < 0.05$) for the interaction cultivar*harvest date for figures A, B, C, D, E and F were 0.80, 0.81, 0.009, 0.009, 0.006 and 0.22, respectively. Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$. CHD stands for Commercial Harvest Date.

3.3. α-Farnesene metabolism

α-farnesene concentrations remained constant in 'Blanquilla' pears as the fruit ripened on-tree (100 μmol kg^{-1}; Fig. 4A). In contrast, an increase, but not statistically significant, from 80 to 160 μmol kg^{-1} was observed in 'Flor d'Hivern' pears from CHD-5 to CHD+4. CTol 281 content in 'Flor d'Hivern' pears also slightly increased in relation to the harvest date whereas in 'Blanquilla' pears this compound decreased by 2-fold from the earliest to the latest harvest (Fig. 4B).

PcAFS1 gene expression was differentially regulated as harvest dates progressed depending on the cultivar. A clear up-regulation was found in 'Blanquilla' pear, especially from CHD-5 to CHDin, while no clear trend was observed for 'Flor d'Hivern' pears (Fig. 4C).

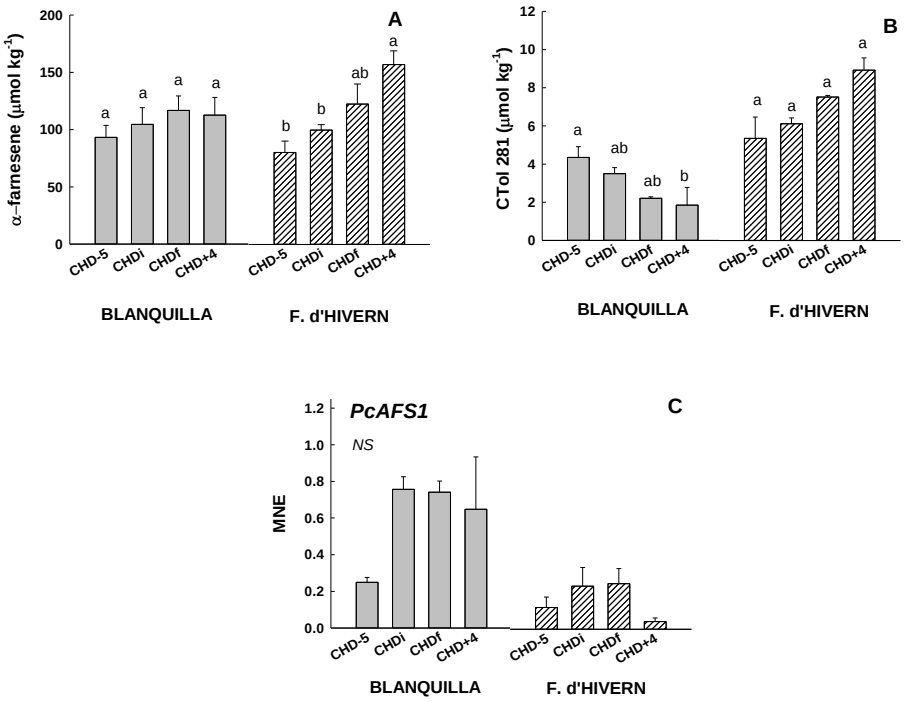

Figure 4: α-Farnesene content (A), its oxidation products CTols (B) and *PcAFS1* gene mean normalised expression (C) for 'Blanquilla' (grey bars) and 'Flor d'Hivern' (striped bars). Error bars represent the standard error of the mean (n=3). LSD values ($p < 0.05$) for the interaction cultivar*harvest date for figures A, B and C were 74.94, 1.99 and 0.34, respectively. Means with

the same letter for each cultivar are not significantly different at $p \leq 0.05$. CHD stands for Commercial Harvest Date.

3.4. Antioxidants and oxidative-related changes

Although 'Flor d'Hivern' pears exhibited higher antioxidant potential than 'Blanquilla' pears (Fig. 5A), harvest date had no effect for either cultivar. A similar trend was found for total ascorbate contents that were higher in 'Flor d'Hivern' pears but not affected by harvest date (Fig. 5B).

LOX activity was nearly 2-fold higher in 'Flor d'Hivern' than in 'Blanquilla' pears being about 15 UA mg^{-1} protein compared to 8 UA mg^{-1} protein in ''Blanquilla' pears, again not affected by harvest date (Fig. 5C). Finally, an inverse behaviour was found for CAT activity that was slightly higher in 'Blanquilla' than in ''Flor d'Hivern' pears regardless of harvest date (Fig. 5D).

An interesting behaviour was recorded for PPO and POX activity in 'Blanquilla' pears. In particular, PPO activity in 'Blanquilla' pears was significantly higher at earlier harvest (CHD-5; 550 UA mg^{-1} protein) if compared to the following harvests (200 UA mg^{-1} protein). In contrast, in 'Flor d'Hivern' pears and despite higher values at CHDi, PPO activity was not affected by harvest date. (Fig. 5E).

POX activity values in 'Blanquilla' pears were almost 500 UA mg^{-1} protein at the earliest harvest date (CHD-5) and 2 to 3-fold lower ($p < 0.05$) in the successive harvest (Fig. 5F). No effect of harvest date was detected for 'Flor d'Hivern' pears, activities ranging from 210-350 UA mg^{-1} protein.

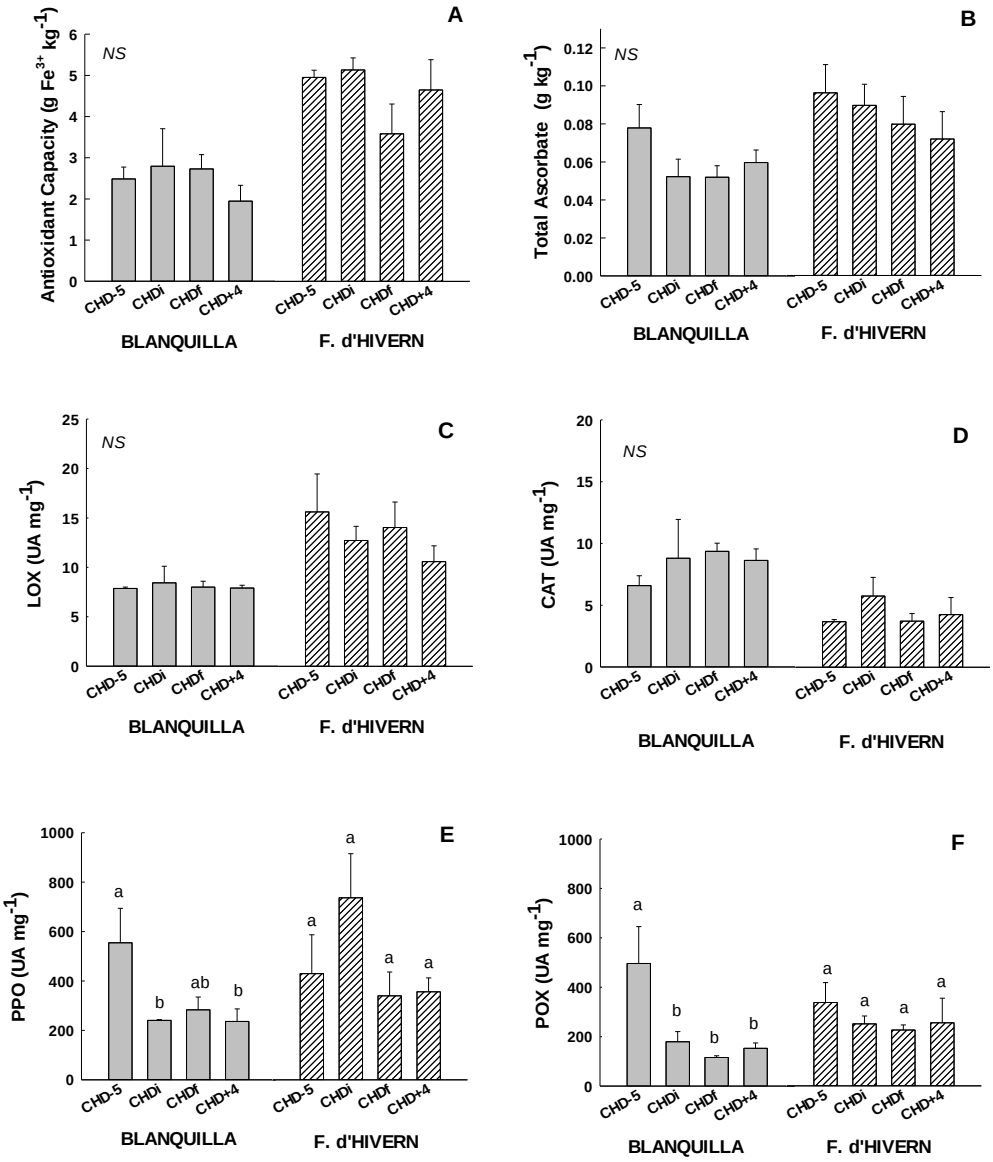

Figure 5: Total antioxidant capacity (A), total ascorbate (B), LOX activity (C), CAT activity (D), PPO activity (E) and POX activity (F) for 'Blanquilla' (grey bars) and 'Flor d'Hivern' (striped bars). Error bars represent the standard error of the mean (n=3). LSD values ($p < 0.05$) for the interaction cultivar*harvest date for figures A, B, C, D, E and F were 0.93, 0.02, 3.29, 2.57, 198.72 and 123.39, respectively. Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$. CHD stands for Commercial Harvest Date.

3.5. Characterisation of fructose and sorbitol content at harvest

Sorbitol metabolism was different between cultivars (Figure 6). In 'Blanquilla' pears, a lower sorbitol content (*ca.* 15 g kg^{-1}) was observed at CHD-5 compared with the other harvest (*ca.* 23 g kg^{-1}; Fig. 6A). In contrast, no significant differences were observed for 'Flor d'Hivern' pears where values remained relatively constant at 13 g kg^{-1} for all harvest dates, meaning about 1.2-1.8-fold lower than in 'Blanquilla' pears. The lower sorbitol contents in 'Flor d'Hivern' pears were associated with significant up-regulation of *PcSDH* especially at CHD+4 (Fig. 6C).

Fructose contents were similar for the cultivars and generally remained unchanged (*ca*. 30 g kg^{-1}) during on-tree fruit ripening (Fig. 6B).

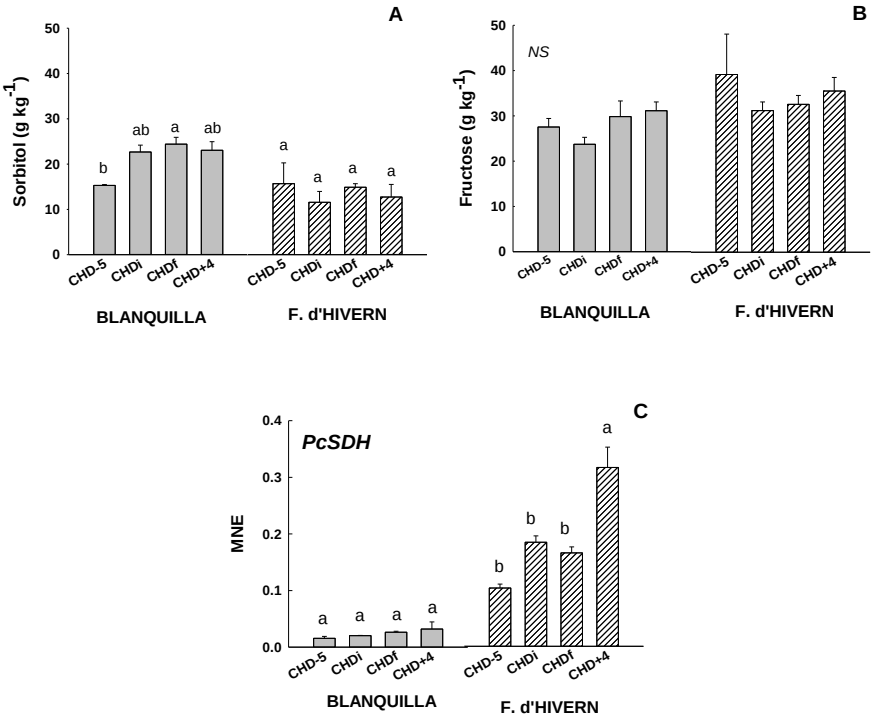

Figure 6: Sorbitol content (A), fructose content (B) and *PcSHD* gene expression (C) for 'Blanquilla' (grey bars) and 'Flor d'Hivern' (striped bars) among the different harvest dates. Error bars represent the standard error of the mean (n=3). LSD values ($p < 0.05$) for the interaction cultivar*harvest date for figures A, B, and C were 6.58, 9.04 and 0.04, respectively. Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$. CHD stands for Commercial Harvest Date.

4. Discussion

4.1. Differences in symptom development and its relationship with the initial harvest date indicate that distinct types of superficial scald

The way by which the scald disorders appeared for the two cultivars was clearly different (Figure 1). 'Blanquilla' pear is highly susceptible to scald and it is commonly used as the model for this storage disorder in a summer-type pears. On the other hand, 'Flor d'Hivern' is a local cultivar, which does not produce ethylene even after long periods of cold storage (winter-type pear behaviour) but also has very high susceptibility to scald (Figure 1). In 'Blanquilla' pears, disorder incidence depended on the initial fruit maturity at harvest and was expressed only after removal from cold storage. In contrast, disorder incidence in 'Flor d'Hivern' cultivar developed during cold storage and regardless of initial harvest date.

Such a behaviour in 'Blanquilla' pears is in accordance with previous studies (Calvo et al., 2015; Villalobos-Acuña et al., 2011) and with the idea that scald development in pome fruit is strongly determined by the fruit physiological maturity at harvest (Calvo et al., 2015). It is directly linked to the fruit capacity to ripen on-tree (Lindo-García et al., 2019) and to the fact that ethylene may trigger specific metabolic changes ultimately associated to scald development. In contrast, the different pattern of scald development observed in 'Flor d'Hivern' pears, shows that the etiology of scald is cultivar dependent and that the same symptoms (brown patches on the fruit surface) can result from multiple and different metabolic shifts yet always induced by cold storage. It is unlikely that the symptoms observed in 'Flor d'Hivern' refer to other disorder such as senescent scald since the fruit susceptibility was not dependent on the maturity stage at harvest and the symptoms exclusively affected the fruit surface without altering the flesh or the fruit taste. To further elucidate the physiological and molecular basis of scald development in these two cultivars, as well as to identify putative markers at harvest, targeted compounds and genes, previously identified as playing a key role in the development of scald (Busatto et al., 2018, 2014; Lindo-García et al., 2019) were analysed in fruit from both cultivars and different maturities.

4.2. Ethylene related markers may predict scald in 'Blanquilla' pears but not in 'Flor d'Hivern'

The ability of 'Blanquilla' pears to ripen on-tree, was clearly mediated by the increasing levels of ACC resulting from higher ACS enzyme activity (Fig. 3A and

Fig. 3C, respectively), that is the key factor limiting ethylene metabolism (Yang and Hoffman, 1984). *PcACO1* gene expression in this cultivar remained down-regulated during on-tree ripening (Fig.3F) and hence did not explain the differences observed for the kinetics of ethylene production among harvests. In 'Blanquilla' fruit, ACS and ACC were positively correlated with disorders incidence (r^2 = 0.634 and 0.709, respectively; $p \leq 0.05$) thereby confirming that the capacity of the fruit to ripen or at least to produce ethylene upon harvest may be a good indicator of the fruit susceptibility to scald. However, further studies are still needed to determine the critical ACC and ACS thresholds at harvest below which 'Blanquilla' pears may not develop scald during storage.

The specific behaviour of 'Flor d'Hivern' pears regarding ethylene metabolism revealed a higher regulatory complexity. In this cultivar, ACC levels remained very low during on-tree ripening regardless of the harvest date, a result that likely explain the incapacity of these fruit to produce ethylene upon harvest. However, and despite of these low ethylene rates, ACS activity was not negligible and ACO activity was even higher than that observed in 'Blanquilla' pear. Since the low levels of ACC were not associated to an ACS inhibition, increased ACO activity and/or higher synthesis of MACC (Fig.3B), we may hypothesize that ACC depletion in this cultivar may be regulated up-stream (Van de Poel and Van Der Straeten, 2014). The lack of ethylene production in 'Flor d'Hivern' may also explain the lack of fruit softening at different harvest dates (Table 1). Accordingly, further studies determining the roles play by methionine and S-adenosylmethionine in the regulation of ethylene production in 'winter pears' are needed to better understand this specific behaviour.

Concomitantly, a clear up-regulation of *PcACS1* and *PcACO1* gene expression likely due to ACC deprivation was also observed in 'Flor d'Hivern' pears as harvest dates progressed. As 'Flor d'Hivern' pear did not produce ethylene nor accumulate ACC, we may speculate that some alterations at the post-translation level are responsible for the lack of association between gene expression and enzyme activity during 'Flor d'Hivern' on-tree ripening.

Our results also show that compounds related to ACC metabolism in 'Flor d'Hivern' pears, in contrast to 'Blanquilla' pears, were not related to the susceptibility of this pear cultivar to scald. The results indicate that scald development in 'Flor d'Hivern' pears was ethylene independent, since this cultivar behaves like a non-climacteric fruit, hence in agreement with previous studies (Busatto et al., 2018; Karagiannis et

al., 2018; Rao et al., 1998) and further suggesting that others regulatory mechanism triggered by cold stress are likely involved in the development of this disorder. Besides, this is the first study showing that a non-climacteric like pear cultivar was very susceptible to superficial scald. That said, it is important to note that albeit not strictly being ethylene dependent, 1-MCP treatment controls scald in 'Flor d'Hivern' (Dupille, E.; personal communication) as also does in 'Blanquilla' pears (Larrigaudière et al., 2019).

4.3. *PcAFS1* as a key marker of scald sensitivity of 'Blanquilla' pears at harvest

The accumulation of α-farnesene and its oxidation into CTols has for long time been recognized to be involved in scald development either as a causal agent (Lurie and Watkins, 2012) or as an intermediate or signalling process (Busatto et al., 2014).

In our study, no clear relationships were found between α-farnesene, CTol281 levels and the differences in disorder incidence for the different harvest dates for any of the cultivars investigated. In 'Blanquilla' pears, however, a clear relationship was found between *PcAFS1* gene expression, the fruit capacity to produce ethylene, and the disorder incidence. These results are in accordance with previous studies which reported that ethylene induces α-farnesene biosynthesis (Ju and Curry, 2000; Lurie and Watkins, 2012) up regulating *PcAFS1* gene expression (Gapper et al., 2006; Pechous et al., 2005). However, considering that the up regulation of *PcAFS1* in this work was not associated to increase α-farnesene levels at harvest, we hypothesize that this regulatory mechanism leading to higher α-farnesene content is evident only during cold storage. That said, changes in *AFS1* gene expression during on-tree ripening were clearly related to scald incidence ($r^2 = 0.593$; $p \leq 0.05$), suggesting then that this parameter may be a potential marker to predict, at harvest, scald susceptibility in 'Blanquilla' pears.

In 'Flor d'Hivern' pears and despite of similar and higher levels of α-farnesene and CTols than in 'Blanquilla' pears, *PcAFS1* gene expression remained low regardless of the harvest date. This result is likely related to the lower ethylene values observed in this cultivar and hence to its inability to trigger *PcAFS1*. Our results suggest then, that in contrast to 'Blanquilla' pears that follow a typical scald model linked to ethylene and α-farnesene accumulation and oxidation, other regulatory mechanisms are likely involved in the regulation of scald disorder in 'Flor d'Hivern' pears.

4.4. The relationship between oxidative-related markers at harvest and superficial scald susceptibility

Given that scald is known to be the result of an oxidative process (Lurie and Watkins, 2012), antioxidants may play a decisive role in scald prevention. Ascorbate for instance was found to play a key role to explain the differences in scald susceptibility between pear cultivars (Larrigaudière et al., 2016). In accordance, a clear relationship was found in this work in 'Blanquilla' pears between the initial levels in ascorbate within the fruit skin and the disorder incidence after cold storage. Combined with the lower POX activity observed after CHDi, these results might explain, at least in part, the increasing scald susceptibility observed in 'Blanquilla' pears in relation to the fruit maturity at harvest. The others parameters such as global antioxidant potential, LOX and CAT activities seem to play a secondary role, in the same way that PPO activity at harvest is unlikely to act as a limiting factor.

The results obtained in 'Flor d'Hivern' pears were again more complex. Although presenting higher initial antioxidant potential and ascorbate levels, higher disorder incidence was observed at all maturity stages. Similar results were reported in a previous study with 'Beurre d'Anjou' pears, where the authors suggested that the initial antioxidant potential of the fruit was not directly related to the capacity of the fruit to develop superficial scald (Calvo et al., 2015). However, the higher POX and especially LOX activity observed in this cultivar, compared with 'Blanquilla', may suggest an enhanced lipid peroxidation as previously described in Japanese pear (Li and Wang, 2009), and hence partially explaining the development of this disorder.

In contrast to 'Blanquilla' pears that exhibited a typical scald disorder associated to ethylene and α-farnesene metabolism, scald-like disorders in 'Flor d'Hivern' seem to be rather related to a classical chilling injury damage in which membrane integrity plays a predominant role. This is further supported by the fact that scald in 'Flor d'Hivern' occurs after short storage durations and becomes evident even during cold storage (Figure 1).

4.5. The involvement of sorbitol and related enzymes with superficial scald susceptibility

Considering these last results and the new information generated in the recent articles of Busatto et al. (2018) in apples, we also analysed the role that cryoprotectants such as sorbitol may play in this context.

Only slight differences in sorbitol and fructose contents were found among harvest dates for the two cultivars (Fig.6A and 6B). Nevertheless, sorbitol contents were lower and fructose contents slightly higher in 'Flor d'Hivern' compared with 'Blanquilla'. Accordingly, these results suggest that 'Flor d'Hivern' already had lower capacity to prevent chilling damage at harvest, since higher concentrations of this compound have been shown to increase the resistance to chilling injury in model species (Busatto et al., 2018). Furthermore, a sharp up-regulation of *PcSDH* was observed in 'Flor d'Hivern' but not in 'Blanquilla'. The higher conversion of sorbitol into fructose, a phenomenon likely occurring during cold storage, may further determine the storage potential of this cultivar limiting its fruit resistance to chilling injury. Combined with the increased peroxidative problems showed earlier, the lack in cryoprotectants in 'Flor d'Hivern' likely determined the higher susceptibility of this cultivar to develop the disorder.

5. Conclusions

The results from this study demonstrate that superficial scald among different pears cultivars with distinct ripening patterns may have different etiologies. Comparing the concentration of ethylene-related or oxidative compounds at harvest of two highly susceptible cultivars, we clearly distinguished two key casual features involved in scald development. The first one, associated to the action of ethylene on ripening and up-regulation of *PcAFS1* gene expression, was only noticeable when the fruit were able to initiate fruit ripening on-tree. This is the case of 'Blanquilla' pears for which ACC concentrations, ACS enzyme activity and *PcAFS1* expression level increased as fruit ripened on-tree in parallel to scald and thereby showing potential to be used at harvest as markers of the fruit susceptibility to scald. In the case of 'Flor d'Hivern', a typical winter pear cultivar, scald development was in a first instance ethylene independent but clearly associated to higher LOX and POX activities, that likely promote membrane peroxidation. This increase in membrane peroxidation coupled to a reduced content of specific compounds generally acting as cell cryoprotectants, such as sorbitol, may explain the development of the disorder in this pear cultivar.

6. Author's contribution

CL and JGB conceived and designed the experiment. VLG and DU performed all quality and biochemical measurements whereas JGB and CLe did and analysed the

gene expression analysis. VLG and CL wrote the article and all remaining authors contributed in improving and revising the manuscript.

7. Declaration of Competing Interest

All authors declare no conflict of interest.

8. Acknowledgments

This work has been supported by the CERCA Programme/Generalitat de Catalunya, and the Ministerio de Economía y Competitividad / MINECO (grant AGL2017-87923-R). Thanks are also given to the Department d'Empresa i Coneixement de la Generalitat de Catalunyafor the predoctoral fellowship to VLG (2019FI-B2-00218). We are grateful to Elisabet Duaigües for technical support and Dr. Núria Vall-llaura for her assistance with the gene expression analysis

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Supplementary Table 1: Primers used for quantitative PCR

Gene	Annotation	Oligonucleotide sequences	Target gene	Metabolic pathway/Biological function
PcACS	Aminocyclopropane-1-carboxylic acid synthase	F5′-ATGCTGGCTTGTTCTGTTGG-3′ R5′-AGGTTCCGTGCAATGACAAG-3′	PCP011500	Ethylene metabolism
PcACO	Aminocyclopropane-1-carboxylic acid oxidase	F5′-AAGGTCAGCAACTACCCTCC-3′ R5′-TGTCATCCTGGAAGAGCAGG-3′	PCP011683	
PcAFS1	α-farnesene synthase	F5′- GAAAACTAGGCCTCGCGAAC-3′ R5′- TTCGATAGCTGCAATGCCGT-3′	PCP028486	α-farnesene metabolism
PcLOX	Lipoxygenase	F5′- CTTCAACGGAGAATCAGGCG -3′ R5′- TCGGTTATGTCATCGAGGGG-3′	PCP002320	Antioxidant capacity
MdPPO	Polyphenol oxidase	F5′-CCTACTCACAAAGCCCAAGC-3′ R5′-CCTCCAAGACCAAGAAGCAC-3′	PCP039035	
MdSDH	Sorbitol dehydrogenase	F5′-ATGGTCACAGCCATTGGTCA-3′ R5′-ACCTTGTCCTTGCCCAGAAG-3′	PCP002232	Sorbitol Metabolism
Md8283	Housekeeping	F5′-CTCGTCGTCTTGTTCCCTGA-3′ R5′-GCCTAAGGACAGGTGGTCTATG-3′	PCP030439	-

CAPÍTULO 5

Unravelling the cold-induced regulation of ethylene and α-farnesene and its involvement with the development of scald-like disorders in different pear cultivars

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Postharvest Biology and Technology, resubmitted on 9th September 2020

Abstract

To better understand the cold-induced regulation of scald-like disorders in pears and the specific roles played by ethylene and α-farnesene, three pear cultivars with different patterns of ethylene production and chilling requirement were used in this study. Fruit were treated with 1-MCP (ethylene inhibitor) and Lovastatin (α-farnesene inhibitor) and stored at -0.5 °C and 90 % RH during 6 months. Changes in targeted metabolites, enzymes and genes were monitored periodically up to 120 d of storage and superficial scald incidence was assessed after this time and after 180 d of cold storage. 1-MCP treatment induced in the three cultivars a down-regulation of *PcACS1*, *PcACO1*, *PcERF1* and *PcAFS1* gene expression, but also a significant up-regulation of *PcETR*1 and *PcEIN2* that led in all cases to the inhibition of the disorder incidence. In contrast, Lovastatin treatment caused diverse molecular or biochemical responses depending on the cultivar. In 'Blanquilla' pears, this treatment completely inhibited superficial scald reinforcing the idea that ethylene-α-farnesene interaction plays a decisive role in this specific cultivar. In contrast to 1-MCP, Lovastatin treatment did not control the disorder incidence in 'Flor d'Hivern' pears. Inversely, 1-MCP inhibited the development of the disorder, showing then that the inhibition of ethylene biosynthetic and signalling pathway may control superficial scald even in cultivars producing very low or undetectable ethylene levels. Finally, the inefficacy of both treatments to prevent the disorder development in 'Conference' pears, suggests the existence of a disorder different from that observed for the other cultivars whose biochemical basis remain unknown. Collectively our results show that the regulatory processes triggered by cold stress in pears are complex and cultivar dependent.

Keywords: 1-MCP, cold induction, Lovastatin, storage, superficial scald.

1. Introduction

Low-temperature storage is a common postharvest practice aiming to prolong the storage life and then the availability of pears in the market (Saquet, 2019). As for many other fruit, low-temperature storage can however lead to the appearance of chilling injury (CI) disorders (Benichou et al., 2018; Ma and Chen, 2003). Superficial scald is by far one of the main CI of pears accounting for important postharvest losses worldwide (Lurie and Watkins, 2012; Wang and Dilley, 1999). This physiological disorder manifests as brown-dark patches on the fruit skin, yet the susceptibility and severity of the symptoms can largely vary among cultivars (Fig.1; Larrigaudière et al., 2016; Lindo-García et al., 2020) and within each cultivar depending on the fruit maturity at harvest (Calvo et al., 2015; Lindo-García et al., 2020). Previous studies have characterised superficial scald or scald-like disorders in pears both at the morphological and biochemical level (Lindo-García et al., 2020; Zoffoli et al., 1998). Generally, symptoms are visible in most cultivars (i.e. 'Blanquilla', 'Abate Fetel', 'Packham's') upon rewarming and after relatively long periods of cold storage (Calvo et al., 2015; Larrigaudière et al., 2019, 2016), yet for some cultivars (i.e 'Flor d'Hivern') symptoms can appear even during cold storage (Lindo-García et al., 2020).

The most accepted theory to explain scald development relates the disorder to the formation and oxidation of α-farnesene into conjugated trienols (Farneti et al., 2015; Mir et al., 1999; Rowan et al., 2001). Under this scenario, ethylene plays a key role by controlling the production of α-farnesene via an up-regulation of α-farnesene synthase gene (*AFS*) (Gapper et al., 2006; Lurie et al., 2005; Pechous et al., 2005). This said, several studies suggest that α-farnesene may also accumulate independently of ethylene, directly in response to cold stress, but to a different extent depending on the cultivar (Calvo et al., 2015; Larrigaudière et al., 2019; Lindo-García et al., 2020). Such response in cold may be explained by the fact that terpenes such as α-farnesene are induced in plants under abiotic stress conditions (Holopainen and Gershenzon, 2010; Torregrosa et al., unpublished) in an attempt to stabilize membranes and prevent the cold-induced cell disruption.

The involvement of either ethylene or α-farnesene in scald development is further sustained by the fact that treatments with the ethylene inhibitor 1-methylcyclopropene (1-MCP) or with Lovastatin (a specific inhibitor of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, HMG-CoA reductase) clearly inhibit scald development in apples and pears (Busatto et al., 2014; Giné-Bordonaba

et al., 2020; Ju and Curry, 2000a; Larrigaudière et al., 2019). Likewise, the oxidative nature of the disorder is also evident since treatments with synthetic antioxidants (i.e. diphenylamine) clearly control the appearance of the disorder without altering ethylene metabolism (Karagiannis et al., 2018).

Albeit the ethylene / α-farnesene theory is still valid, recent studies pointed out that others multiple complex metabolic changes are ultimately responsible for the development of the disorder. For instance, the oxidation of specific phenolic compounds (i.e. chlorogenic acid) via polyphenol oxidase (PPO) or the metabolism of cryoprotectants (i.e. sorbitol), volatiles or antioxidants seem to be also crucial pathways associated with the development of the disorder (Busatto et al., 2018, 2014; Giné-Bordonaba et al., 2020; Wang et al., 2018).

Little information is currently available about how cold storage may trigger these metabolic changes finally leading to the development of superficial scald. Accordingly, this study aimed to investigate the cold-induced regulation of both ethylene and α-farnesene biosynthesis, both at the biochemical and molecular level. Specific inhibitors (1-MCP and Lovastatin) were used to define the way by which these two compounds participate individually or collectively to the development of scald-like disorders in the three studied cultivars.

2. Material and methods

2.1. Plant material and experimental design

'Blanquilla', 'Conference' and 'Flor d'Hivern' pears (*Pyrus communis* L.) were chosen based on their differential susceptibility to skin browning disorders (Lindo-García et al., 2020) but also given their different ethylene production pattern and chilling requirements. Fruits were harvested at a firmness values of 57.1 N, 62.5 N and 49.3 N, respectively, on a commercial orchard near Lleida (Catalonia, Spain). Harvest date corresponded to the commercial harvest date (CHD; about 125, 135 and 173 d after full bloom for 'Blanquilla', 'Conference' and 'Flor d'Hivern', respectively), based on standard local recommendations (Lindo-García et al., 2020; Torregrosa et al., 2019).

2.2. Treatments

Immediately after harvest, fruit from each variety were divided into three different batches of 240 fruit each. One batch (240 fruit) was placed in a sealed plastic container and treated with 300 nL L^{-1} 1-MCP during a minimum of 18 h at 0 °C and using the product Smartfresh™ (Agrofresh Inc.). Lovastatin treatment was done on 240 fruit by dipping them into a 1.25 mmol L^{-1} solution (Giné-Bordonaba et al., 2020) during 2 minutes. The lovastatin formulation was prepared by dissolving 30.3 g of Lovastatin (98 %), 240 g of sunflower oil, 240 g of glycerol and 720 g of Tween-80 in 2.4 L of hot water and then adding water until 60 L. Based on preliminary trials, none of the additional components added to the lovastatin formulation influenced by themselves superficial scald incidence in apples or pears. Finally, a batch of untreated fruit served as a control. After treatments, fruit were stored at -0.5 °C and 90 % RH until further physiological or biochemical analyses.

2.3. Determination of α-farnesene (AF) and conjugated trienols (CTs)

AF and CT_{281} were analysed as described by Anet (1972) with some modifications (Larrigaudière et al., 2019). At harvest and after 7, 15, 30, 60 and 120 d of cold storage, 9 fruit of each treatment were removed and a strip of peel was removed from the equatorial zone of each fruit and 6 discs (10 mm diameter) prepared using a cork borer. The discs were immersed in 5 mL of HPLC grade hexane for 10 min with constant stirring and then the solution was filtered and mixed with hexane until a final volume of 5 mL. Measurements were performed calibrating first the equipment with HPLC grade hexane. Absorbance at 232 nm (α-farnesene), 281 and 290 nm (conjugated trienol - CT_{281}) were recorded using a UV-spectrophotometer (1001 Plus, Milton Roy, USA). Concentrations of α-farnesene and conjugated trienols were calculated using the molar extinction coefficients E_{232nm}= 27,700 for α-farnesene and $E_{281\text{-}290nm}$ = 25,000 for conjugated trienols and the results expressed as μmol kg^{-1} peel.

2.4. Ethylene production

Ethylene production (nmol kg^{-1} s^{-1}) was measured after 60 d of cold storage as described by Giné-Bordonaba et al. (2014). Briefly, 2 fruit per replicate and 3 replicates per treatment and removal time were placed in 1.5 L flasks continuously ventilated with humidified air at a flow rate of 1.5 L h^{-1}. Gas samples (1 mL) were taken of effluent air using a 1 mL syringe and injected into a gas chromatograph (CG; Agilent Technologies 6890, Wilmington, Germany) fitted with a FID detector and an Alumina

column F1 80/100 (2 m x 1/8 x 2.1, Tecknokroma, Barcelona, Spain). The oven temperature was 140 °C while the injector and detector were kept at 180 and 280 °C, respectively.

2.5. Determination of 1-aminocyclopropane-1-carboxylic acid (ACC) levels and ACC synthase and ACC oxidase activity

Flesh tissue from 3 individual fruit per replicate and 3 replicates per treatment was frozen in liquid nitrogen at harvest and after 7, 15, 30, 60 and 120 d of cold storage, and kept at -80 °C until further biochemical assays.

1-aminocyclopropane-1-carboxylic acid (ACC) was extracted and analysed as described by Bulens et al. (2011) with some modifications as specified in Lindo-García et al. (2019).

1-Aminocyclopropane-1-carboxylic acid oxidase enzyme (ACO) was extracted as described by Lindo-García et al. (2019). The enzyme activity was analysed as described by Giné-Bordonaba et al. (2017) and results expressed as nmol C_2H_4 kg^{-1} s^{-1} on fresh weight basis.

The extraction and analysis of the activity of 1-aminocyclopropane-1-carboxylic acid synthase enzyme (ACS) was determined as also described by Lindo-García et al. (2019).

2.6. Determination of superficial scald incidence

Scald incidence for each treatment was estimated visually after 6 months of cold storage plus 7 d of shelf life (20 °C) as described by Larrigaudière et al. (2019). Superficial scald incidence was expressed as the percentage of damaged fruit, but also establishing the severity of the damage according to a 0 to 4 scale in which:

S0 = No damaged fruit; S1 = <10% of the skin surface damaged; S2 = <25% of the skin surface; S3 = <50% of the skin surface and S4 = >50% of the skin surface.

The final index was calculated with the following formula.

$$\text{Severity} = \frac{\sum \text{S0x0} + \sum \text{S1x1} + \sum \text{S2x2} + \sum \text{S3x3} + \sum \text{S4x4}}{\text{Total number of fruit}}$$

2.7. RNA extraction and Gene expression analysis

Peel tissue from 3 individual fruit per replicate and 3 replicates per treatment was frozen in liquid nitrogen at harvest and after 15, 30, and 60 d of cold storage, and kept at -80 °C until further molecular assays.

Total RNA was extracted using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, St Louis, MO, USA). RNA quantity was determined spectrophotometrically using a NanoDrop 2000 spectrophotometer (Thermo Scientific) and both absence of contaminant DNA and RNA integrity were assessed after electrophoresis on an agarose gel stained with GelRed™ Nucleic Acid Gel Stain (Biotium, Hayward, CA, USA). First-strand cDNA synthesis was performed with an oligo-dT primer on 1 µg of RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) on a Verity Thermal Cycler 96-wells Fast (Applied Biosystems, Foster City, CA). Gene expression analysis was performed as described by Baró-Montel et al. (2019) using KAPA SYBR® Fast qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, USA) as polymerase master mix and with the following conditions: 95 °C (10 s) followed by 40 cycles of 95 °C (15 s) and 60 °C (1 min). Most of the oligonucleotides used for RT-qPCR analysis were adopted from Busatto et al. (2019), *PcHMGR* and *PcETR1* were adopted from Giné-Bordonaba et al. (2020) and Chiriboga et al. (2013), respectively, and *PcEIN2* was designed using the Primer-BLAST tool (Ye et al., 2012). *Md8283* was used as independent reference gene based on previous studies (Busatto et al., 2019, 2018) but also given the constant expression along cultivars and treatments shown in preliminary trials. The primers used in this study are listed in Supplementary Table 1. Primer efficiency was confirmed to be >90 % using 3-fold cDNA dilutions in triplicate and primer specificity was checked by analyzing the melting curves at temperatures ranging from 60 to 95 °C. A non-template control (NTC) was included using water instead of DNA. Relative gene expression was expressed as Mean Normalized Expression (MNE) and calculated using the method described by Muller et al. (2002).

2.8. Statistical analysis

All data were subjected to analysis of variance (ANOVA) using JMP® 13.1.0 SAS Institute. Comparisons between time samplings and/or treatments for each variety were done by Tukey's test at a significant level of $p \leq 0.05$ (*) and $p \leq 0.01$ (**). Least significant difference values (LSD; $p = 0.05$) for the interaction treatment*samplings of

cold storage were calculated for mean separation using critical values of *t* for two-tailed tests.

3. Results

Important differences in scald-like or superficial scald disorder incidence were found for the different cultivars investigated herein (Fig. 1). In 'Blanquilla' pears, superficial scald incidence was relatively low after 6 months of cold storage (28.3 %; data not shown) but rapidly increased thereafter reaching 95 % after 7 d of shelf-life (Fig. 1D). In contrast, scald-like incidence in 'Flor d'Hivern' was very high already upon removal from cold storage (76.7 %; data not shown) and a slight increase during shelf-life (85.0 % at 7 d, Fig.1E). Conference pears showed little disorder incidence after 6 months of cold storage (13.3 %, data not shown), regardless of initial harvest maturity (data not shown), as well as minor changes in the disorder incidence when the fruit were left to ripen at 20 °C (21.7 % at 7 d, Fig. 1F).

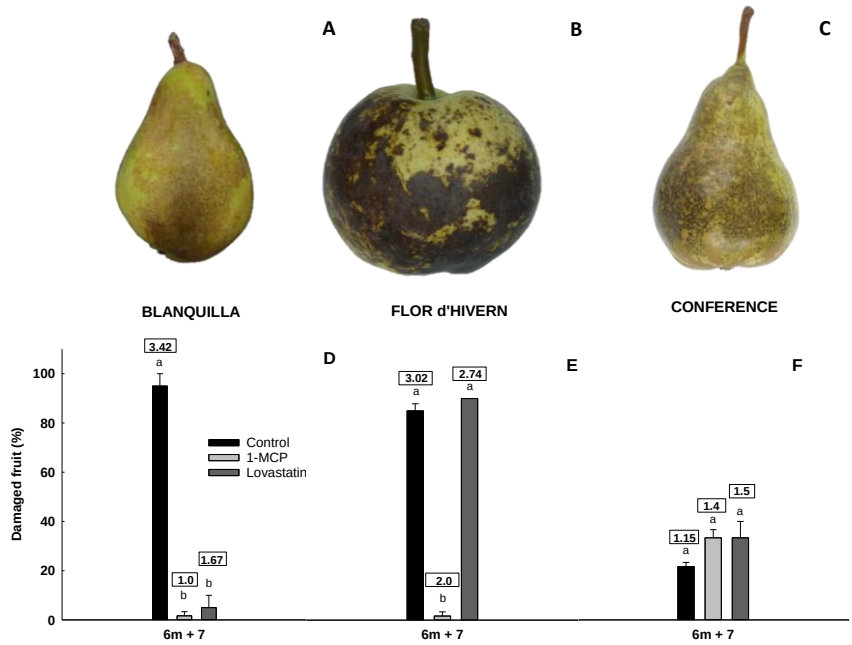

Figure 1: Scald-like disorder appearance and disorder incidence (%) in 'Blanquilla' (A and D), 'Flor d'Hivern' (B and E) and 'Conference' (C and F) pears. Numbers inside boxes in the lower panel indicate severity for each treatment. Error bars represent the standard error of the mean (n = 3). Means with the same letter for each cultivar are not significantly different at $p \leq 0.05$.

In general, the results showing the severity of the disorder paralleled those of the disorder incidence (higher severity associated to higher number of damaged fruit), except for 1-MCP treated 'Flor d'Hivern' pears, that presented very low incidence yet relatively high severity index after 6 months of cold storage.

3.1. Biochemical and molecular events involved in the development of scald-like disorder in 'Blanquilla' pear

3.1.1. Treatment effect on scald-like disorder incidence

Clear differences between treatments were observed in 'Blanquilla' pears after 4 (data not shown) and 6 months of cold storage (Fig. 1D). 1-MCP treatment completely inhibited the disorder incidence in this cultivar (only 1.67 % of damaged fruit after 6 months of cold storage plus 7 d of shelf life) while control fruit showed an incidence of 95 %. Lovastatin treatment also effectively reduced superficial scald incidence (5% of affected fruit) after cold storage and shelf-life.

3.1.2. Cold-induced regulation of ethylene production and ACC metabolism in untreated and treated 'Blanquilla' pears

The patterns of ethylene production in untreated fruit and Lovastatin-treated fruit after 2 months of cold storage were similar, reaching the climacteric peak at 4 d of shelf-life (Suppl. Fig. 1). In contrast, 1-MCP clearly inhibited the fruit ethylene. The inhibition of ethylene production observed in 1-MCP-treated pears after removal from cold storage (Suppl. Fig. 1) was associated to a down-regulations of *PcACS1* and *PcACO1* genes occurring throughout cold storage (Fig. 2 and Suppl. Fig. 3A and 3D), and a decrease of the respective enzyme activities, especially ACO (27- and 9.5-fold lower ACO activity in 1-MCP-treated fruit compared to control fruit at day 60 and 120, respectively; Fig. 2 and Suppl. Fig. 2D). On the other hand, Lovastatin treatment did not affect the gene expression of either *PcACS1* or *PcACO1* (Fig. 2 and Suppl. Fig. 3A and 3D) but rather significantly enhanced ACO enzyme activity at day 120, showing values more than 2-fold higher in Lovastatin-treated than in untreated fruit (Fig. 2 and Suppl. Fig. 2D). No clear pattern was observed for ACC content in any treatment until day 30. From this day, 1-MCP-treated fruit showed a decrease in ACC content, reaching values 3.5- and 4.1-fold lower if compared to untreated and Lovastatin-treated fruit, respectively (Fig. 2).

Ethylene signalling and perception was also differentially affected by the treatments. 1-MCP-treated fruit exhibited a slight up-regulation of *PcETR1* after 15 d of cold storage (Fig. 2 and Suppl. Fig. 3J) and of *PcEIN2* later at 30 and 60 d (Fig. 2 and Suppl. Fig. 3G). A down-regulation of the ethylene response factor, *PcERF1*, was also observed in 1-MCP-treated fruit if compared to untreated fruit at day 60 (Fig. 3 and Suppl. Fig. 2M). Inversely to 1-MCP, Lovastatin treatment led to a slight down-regulation of *PcEIN2* and *PcETR1* during all the storage period (Fig. 2 and Suppl. Fig. 3G and 3J) and a slight and transitory up-regulation of *PcERF1* at 15 and 30 d compared to untreated fruit (Fig. 2 and Suppl. Fig. 3M).

3.1.3. Regulatory processes related to α-farnesene metabolism in 'Blanquilla' pears

A clear relationship between α-farnesene, CT_{281} levels and superficial scald incidence was observed in 'Blanquilla' pear. 1-MCP inhibited the accumulation of α-farnesene, yet showing a slight increase from 64 to 114 μmol kg^{-1} during cold storage and values 7.4-fold lower than untreated fruit after 120 d at -0.5 °C (Fig. 2). A similar tendency was observed for CT_{281} values, where 1-MCP-treated fruit showed basal levels compared to control fruit (Fig. 2). The lower values of these metabolites observed in 1-MCP-treated fruit was related to a down-regulation of both *PcHMGR* and *PcAFS1* gene expression (Fig. 2 and Suppl. Fig. 4A and 4D).

Although Lovastatin treatment also caused a clear inhibition of α-farnesene and CT_{281} accumulation throughout cold storage, this treatment did not affect *PcHMGR* and even caused an up-regulation of *PcAFS1* gene expression if compared to untreated fruit (Fig. 2 and Suppl. Fig. 4A and 4D).

Overall, our data show a classical association between ethylene and *PcAFS1* suggesting that ethylene is a key factor involved in the regulation of superficial scald in 'Blanquilla' pears.

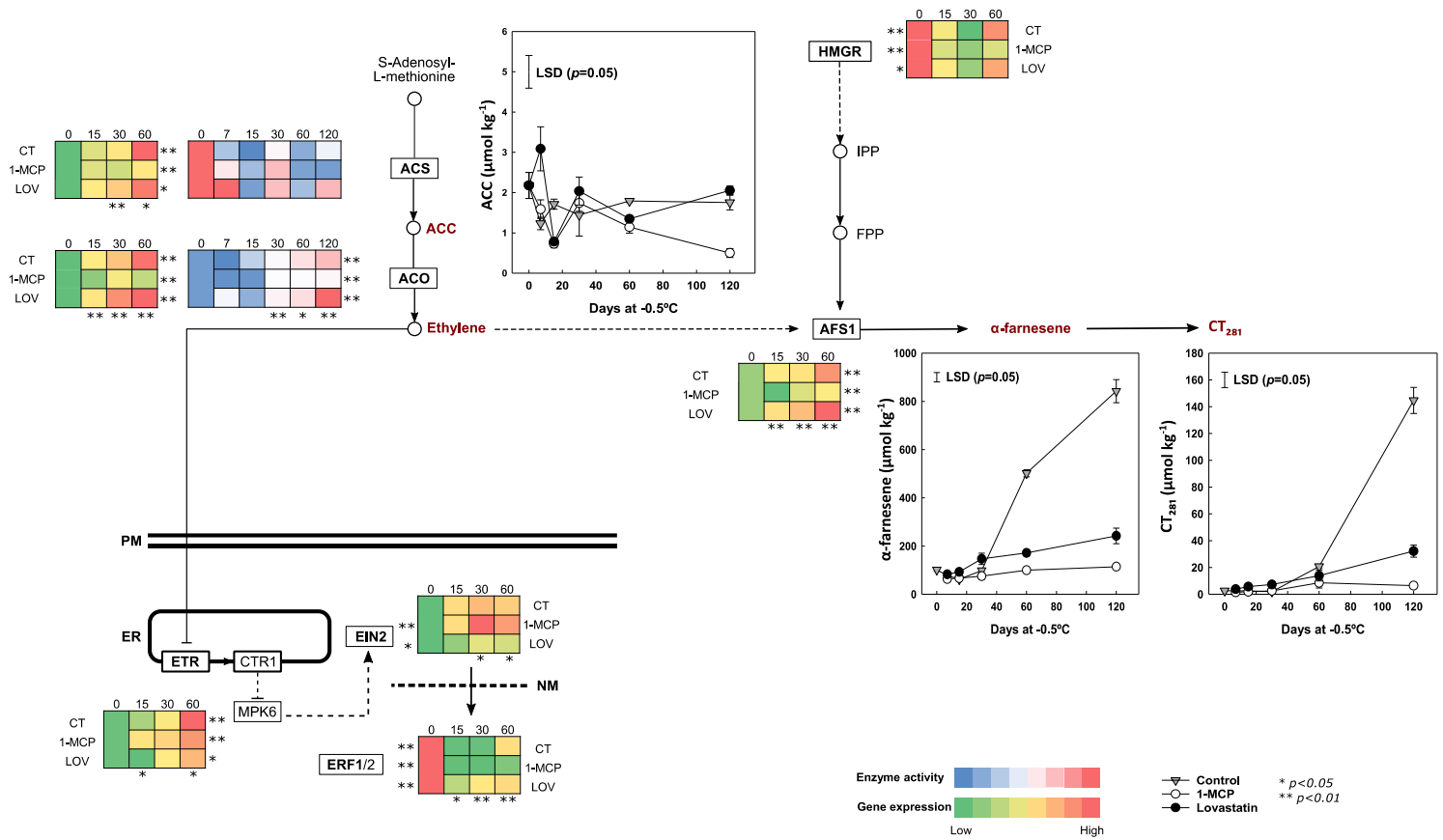

Figure 2: Scheme of the regulatory mechanisms involved in scald development in 'Blanquilla' pears. Error bars represent the standard error of the mean (n = 3). The enzyme activities of ACS and ACO and the gene expression of *PcACS1*, *PcACO1*, *PcETR1*, *PcEIN2*, *PcERF1*, *PcAFS1* and *PcHMGR* are represented as heatmaps where * and ** indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$, respectively, between treatments or sampling points. Single error bar in line plots depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.

3.2. Biochemical and molecular events involved in the development of scald-like disorders in 'Conference' pears

3.2.1. Treatment effect on scald-like disorder incidence

Conversely to the results observed in 'Blanquilla', 'Conference' pear did not showed clear differences in the disorder incidence between treatments. Control fruit exhibited 21.7 % of damaged fruit while 1-MCP- and Lovastatin-treated fruit even showed a higher, yet no significant, disorder incidence (33.3 % of damaged fruit for both treatments; Fig. 1F). Symptoms of the disorder were also slightly different to those observed in 'Blanquilla' fruit (Fig.1). The symptoms in 'Blanquilla' were more diffuse and brown in colour while the symptoms in 'Conference' were darker, less diffuse and seemed not to affect the lenticels. Taken together, these results suggest that superficial scald in 'Blanquilla' and scald-like disorder in 'Conference' are likely two different disorders yet showing similar symptoms.

3.2.2. Cold-induced regulation of ethylene production and ACC metabolism in untreated and treated 'Conference' pears

Control and Lovastatin-treated fruit showed similar ethylene production patterns after 2 months of cold storage, reaching the climacteric peak after 7 d at 20 °C (*ca.* 0.43 nmol kg^{-1} s^{-1}), while 1-MCP treatment completely inhibited the ethylene production upon removing the fruit from cold storage (Suppl. Fig. 1). Similarly to that observed in 'Blanquilla' pears, the ethylene inhibition by 1-MCP was related to lower ACS and especially ACO enzyme activities during storage (Fig.3 and Suppl. Fig. 2B and 2E). This inhibition was also related to a significant down-regulation of both *PcACS1* and *PcACO1* gene expression during cold storage (Fig. 3 and Suppl. Fig. 3B and 3E) and lower ACC content (5.5-fold lower at day 120 if compared to control fruit; Fig. 3). On the other hand, Lovastatin treatment did not affect the transcript levels of *PcACS1* (Fig. 3 and Suppl. Fig. 3B and 3E) nor the ACS or ACO enzyme activities compared to untreated fruit (Fig. 3 and Suppl. Fig. 2B and 2E).

At the signalling and perception level, the results observed in 'Conference' pear were similar to those previously described for 'Blanquilla'. 1-MCP treatment caused an up-regulation of *PcEIN2* gene expression at the beginning of the cold storage (Fig. 3 and Suppl. Fig. 3H) and also a slight up-regulation of *PcETR1* (Fig.3 and Suppl. Fig. 3K). On the contrary, a clear down-regulation of *PcERF1* was observed in 1-MCP-treated 'Conference' pears compared to control fruit (Fig.3 and Suppl. Fig. 3N). A complete

opposite behaviour was found in Lovastatin-treated fruit. In detail, Lovastatin-treated fruit exhibited a down-regulation of both *PcEIN2* and *PcETR1* together with an up-regulation of *PcERF1* gene expression levels in comparison to untreated fruit (Fig. 3 and Suppl. Fig. 3H, 3K and 3N).

3.2.3. Regulatory processes related to α-farnesene metabolism in 'Conference' pear

Conversely to 'Blanquilla', no clear relationship between α-farnesene, CT_{281} levels and scald-like disorder incidence was observed in 'Conference' pear. Higher values of α-farnesene and CT_{281} were observed in control fruit at day 120, suggesting that both 1-MCP and Lovastatin inhibited the accumulation of these metabolites in 'Conference' pears, yet to a lesser extent than in 'Blanquilla' (Fig. 3).

1-MCP clearly down-regulated the expression of *PcAFS1* and impaired the up-regulation of *PcHMGR* at day 60 (Fig. 3 and Suppl. Fig. 4B and 4E). In turn, Lovastatin treatment induced a slight down-regulation of *PcHMGR* and an up-regulation of *PcAFS1* especially at the end of cold storage in comparison to untreated fruit Fig. 3 and Suppl. Fig. 4B and 4E).

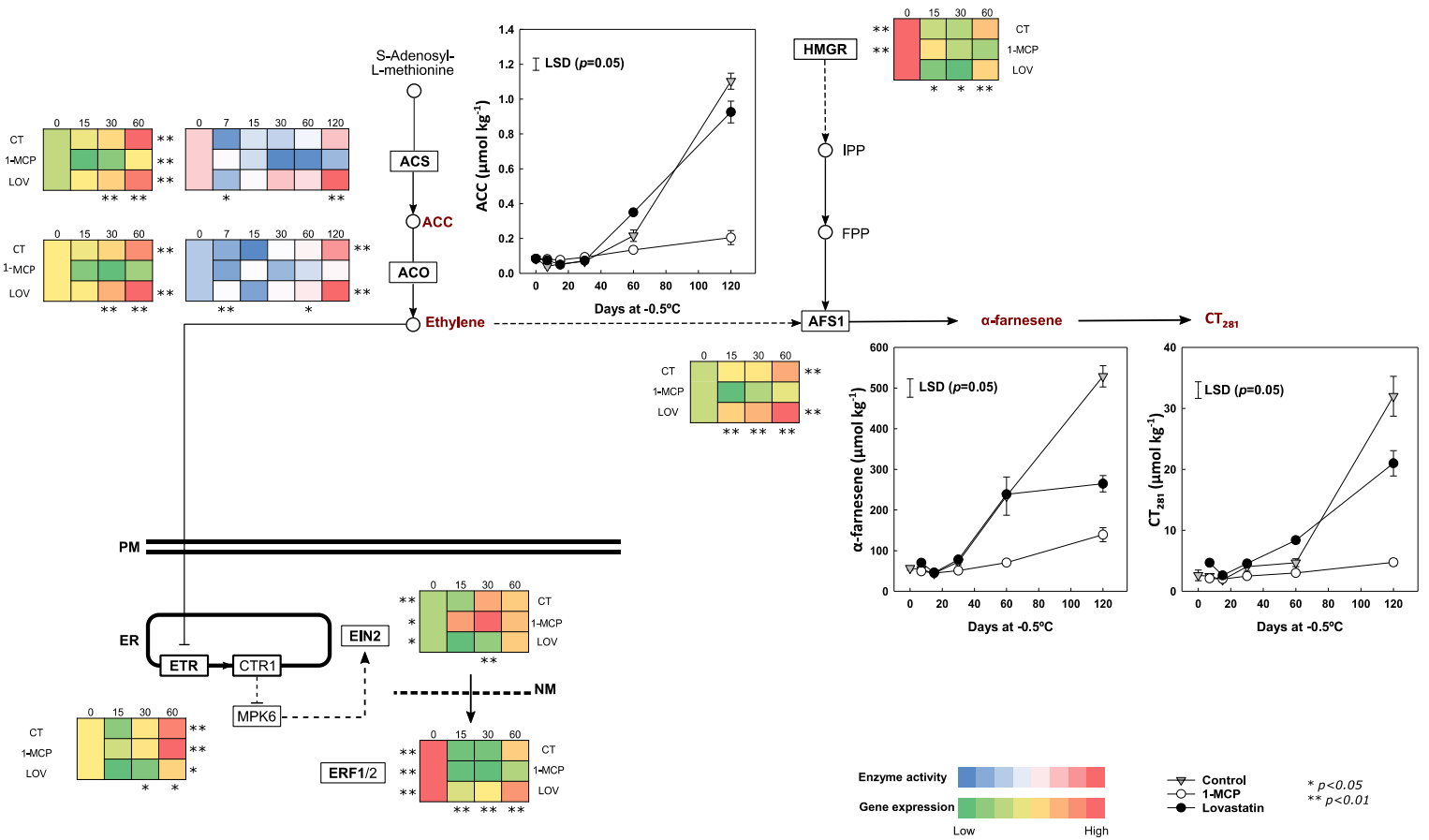

Figure 3: Scheme of the regulatory mechanisms involved in scald development in 'Conference' pears. Error bars represent the standard error of the mean (n = 3). The enzyme activities of ACS and ACO and the gene expression of *PcACS1*, *PcACO1*, *PcETR1*, *PcEIN2*, *PcERF1*, *PcAFS1* and *PcHMGR* are represented as heatmaps where * and ** indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$, respectively, between treatments or sampling points. Single error bar in line depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.

3.3. Biochemical and molecular events involved in the development of scald-like disorders in 'Flor d'Hivern'

3.3.1. Treatment effect on scald-like disorder incidence

Clear differences in superficial scald incidence were observed in 'Flor d'Hivern' pears between treatments. After 6 months of cold storage plus 7 d of shelf life, control fruit showed an incidence of 85 % similar to that observed in Lovastatin-treated fruit (90 %). 1-MCP, in contrast, clearly controlled scald incidence, showing only 2 % of the disorder incidence after 6 months of cold storage plus 7 d of shelf-life (Fig. 2C).

3.3.2. Cold-induced regulation of ethylene production and ACC metabolism in untreated and treated 'Flor d'Hivern' pears

Despite exhibiting a very high incidence of scald-like disorder, this cultivar did not produce detectable amounts of ethylene after 2 months of cold storage (Suppl. Fig. 1). The lack of ethylene production in untreated and Lovastatin-treated fruit were not explained by a repression of either ACS or ACO enzyme activities nor by the expression of their respective genes during storage, since similar levels to that observed in 'Blanquilla' and 'Conference' pears were found in this cultivar. ACC levels increased both in Lovastatin-treated and untreated fruit but the levels reached in control fruit at 120 d were 2.92- and 1.83-fold lower than those observed in 'Blanquilla' and 'Conference', respectively (Fig. 4).

Despite not affecting the fruit ethylene production, 1-MCP treatment induced a clear inhibition of ACO enzyme activity and also of *PcACS1* and *PcACO1* gene expression levels. This said, an increase in ACS activity from day 60 together with limited ACO activity in 1-MCP-treated fruit resulted in enhanced ACC levels from day 60 to 120.

Although not producing detectable amounts of ethylene, the genes involved in the ethylene signalling and perception pathway showed a similar pattern to those observed in 'Blanquilla' and 'Conference' pears. Concretely, 1-MCP-treated fruit showed a time-consistent up-regulation of *PcEIN2* and *PcETR1* (Fig. 4 and Suppl. Fig. 3I and 3L) and a slight down-regulation of *PcERF1* (Suppl. Fig. 3O). On the contrary, Lovastatin treatment caused a down-regulation of *PcEIN2* and *PcETR1* ((Fig. 4 and Suppl. Fig. 3I and 3L) but did not affect the expression level of *PcERF1* (Fig. 5 and Suppl. Fig. 3O).

3.3.3. Regulatory processes related to α-farnesene metabolism in 'Flor d'Hivern' pear

Control and Lovastatin-treated fruit showed a similar pattern of α-farnesene accumulation during cold storage (Fig. 4). A similar tendency was also observed for CT_{281} even though control fruit reached values 1.83-fold higher than Lovastatin treated-fruit after 120 d of cold storage. As observed in the other cultivars, 1-MCP treatment strongly inhibited the accumulation of both α-farnesene and CT_{281}. These results were in agreement with the disorder incidence since a similar scald incidence was observed between control and Lovastatin treatment (85-90 %), while 1-MCP strongly inhibited the disorder incidence (2 %).

From a molecular perspective, both 1-MCP and Lovastatin regulated *PcHMGR* in a similar manner, down-regulating its expression at 30 and 60 d of cold storage if compared to untreated fruit. 1-MCP clearly down-regulated *PcAFS1* gene expression throughout cold storage (Fig. 4 and Suppl. Fig. 4F) in comparison to both untreated or Lovastatin-treated fruit.

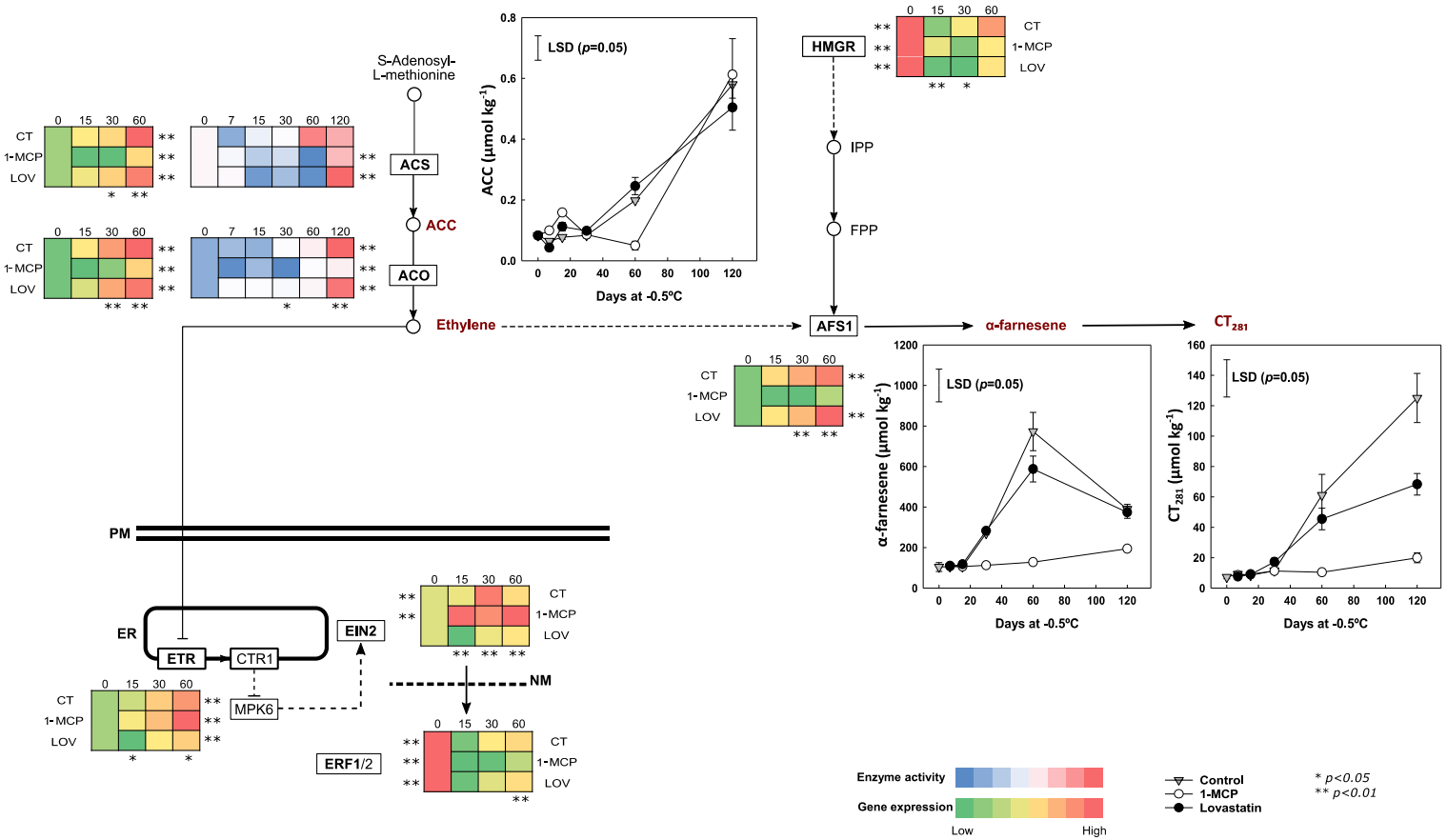

Figure 4: Scheme of the regulatory mechanisms involved in scald development in 'Flor d'Hivern' pears. Error bars represent the standard error of the mean (n = 3). The enzyme activities of ACS and ACO and the gene expression of *PcACS1*, *PcACO1*, *PcETR1*, *PcEIN2*, *PcERF1*, *PcAFS1* and *PcHMGR* are represented as heatmaps where * and ** indicate significant differences at $p \leq 0.05$ and $p \leq 0.01$, respectively, between treatments or sampling points. Single error bar in line plots depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.

4. Discussion

Even though superficial scald is one of the most studied physiological disorders in apples and pears (Calvo et al., 2002; Emongor et al., 1994; Lurie and Watkins, 2012; Xie et al., 2014), its molecular or biochemical basis has been mainly studied after cold storage when the symptoms are visible (Busatto et al., 2018; Gamrasni et al., 2010; Giné-Bordonaba et al., 2020; Villalobos-Acuña et al., 2011; Zhou et al., 2020). Albeit the disorder appears after relatively long-term cold storage, depending on the cultivar and the fruit maturity at harvest (Calvo et al., 2015; Lindo-García et al., 2020), its induction is thought to occur mainly during the first weeks at low temperature (Lurie and Watkins, 2012). Accordingly, our study was directed to better understand these primary events and especially the specific role that ethylene and α-farnesene may play in the induction of superficial scald in different pear cultivars.

4.1. Cold-induced regulation of ethylene and its involvement in superficial scald development

The involvement of ethylene in superficial scald development has been deeply studied over the past decades since this hormone regulates the expression of the α-farnesene synthase 1 (*AFS1*) gene, involved in the last step of the α-farnesene biosynthetic pathway (Lurie et al., 2005; Pechous et al., 2005). Indeed, treatments with the ethylene inhibitor 1-MCP reduce the accumulation of α-farnesene (Isidoro and Almeida, 2006; Larrigaudière et al., 2019; Zhi and Dong, 2018) and is among the most effective treatments to prevent the appearance of the disorder both in apples and pears (Busatto et al., 2018; Calvo et al., 2018; Du et al., 2017). To further understand the role of ethylene in superficial scald development, three different cultivars with known differences in their ethylene production rates were selected in this study (Lindo-García et al., 2020).

'Blanquilla' pear is a typical summer cultivar able to produce ethylene already at harvest (Lindo-García et al., 2019) and highly susceptible to superficial scald (Giné-Bordonaba et al., 2020; Larrigaudière et al., 2019). 'Flor d'Hivern' pears, belong to the winter cultivar type and also develop high incidence of scald-like disorders despite producing very low ethylene levels even after prolonged cold storage (Lindo-García et al., 2020). 'Conference' pears finally, represent an intermediate cultivar requiring short-term cold storage to produce ethylene and much more resistant to the development of scald-like disorders.

Despite their differences in ethylene production, 1-MCP treatment in general led to similar down-regulation of ethylene biosynthetic genes and enzymes in all the cultivars throughout cold storage (Figs. 2, 3 and 4 and Suppl. Fig. 2 and 3), hence consistent with the literature (Busatto et al., 2014; Chiriboga et al., 2013b; Gamrasni et al., 2010; Xie et al., 2016; Zhao et al., 2020). Likewise, a similar 1-MCP effect on the ethylene perception and signalling pathways was observed in all cultivars with treated fruit showing a slight up-regulation of *PcEIN2* and *PcETR1* genes (Figs. 2, 3 and 4 and Suppl. Fig. 3; Chiriboga et al., 2013b; Zhou et al., 2017). By contrast, 1-MCP-treated fruit from the three cultivars studied showed a clear down-regulation of the *PcERF1* gene expression (Figs. 2, 3 and 4 and Suppl. Fig. 3). Such changes in ethylene signalling pathway are likely related to the fact that 1-MCP completely inhibits ethylene production. In this way, the up-regulation of *PcETR1* is likely the result of ethylene deprivation and the up-regulation of *PcEIN2*, that positively interacts with *PcETR1* (Bisson et al., 2009; Bisson and Groth, 2010), a consequence of the regulation of *PcETR1*. The down regulation of *PcERF1* is also likely the consequence of the general inhibition of the ethylene signalling pathway. However, and as *PcERF1* down-regulation was not observed in 1-MCP treated 'Blanquilla' pears after 4 months of cold storage (Giné-Bordonaba et al., 2020), the effect of 1-MCP on this specific gene is likely transitory and only observed during the first months of cold storage.

Overall, our results indicated that the way by which superficial scald is induced is specific for each pear cultivar. In 'Flor d'Hivern' and 'Blanquilla' pears in which 1-MCP completely control the disorder development, superficial scald appeared to be linked to ethylene dependent processes taking place during cold storage. These processes likely play the main determining role in scald development. However, and as previously reported in apple (Karagiannis et al., 2018) and pear fruit (Giné-Bordonaba et al., 2020; Larrigaudière et al., 2019), we cannot discard the involvement of other ethylene independent processes likely associated to fruit acclimation. An ethylene-independent regulation of the disorder was instead observed in 'Conference' pears. Indeed, in this cultivar, 1-MCP effectively inhibited the ethylene production but also slightly enhanced the scald-like disorder incidence (Fig. 1). Similarly, Rizzolo et al. (2015) reported no incidence of superficial scald in 'Conference' pear after 4 months of cold storage and identified two different types of peel disorders (blackening and black speck), which were not inhibited by 1-MCP. Overall, our results are in accordance with the above-mentioned study and suggest that the disorder observed in 'Conference' pear is a scald-like type disorder, yet with

completely different etiology. Further studies at the biochemical and molecular level are needed to better understand and characterise the disorder in this pear cultivar.

4.2. Cold-induced regulation of α-farnesene and its involvement in superficial scald development

In addition to ethylene, superficial scald is commonly related to α-farnesene metabolism and to a widely described relationship between ethylene and α-farnesene (Anet, 1972; Giné-Bordonaba et al., 2013; Whitaker et al., 2000). Albeit not working at the molecular level, Lovastatin is an inhibitor of α-farnesene biosynthesis that does not affect the ethylene production (Ju and Curry, 2000b) but effectively controls superficial scald both in apples and pears (Giné-Bordonaba et al., 2020; Ju and Curry, 2000a). This compound hence, is a very interesting tool to understand the specific role that α-farnesene may have on superficial scald development. Several studies have reported that ethylene promotes the α-farnesene biosynthesis by its action on the *AFS1* gene expression (Gapper et al., 2006; Lurie et al., 2005; Pechous et al., 2005; Tsantili et al., 2007). Our results support these findings but also suggest that *AFS1* is directly activated by cold as soon as the ethylene metabolism at the molecular level is active. These results are in accordance to those observed in previous studies (Calvo et al., 2015; Larrigaudière et al., 2019, 2016) and highlight the idea that α-farnesene is synthesized in pears both in response to increased ethylene production but also in a constitutive way determined by the genetic potential of each cultivar and likely induced by cold stress.

In contrast to 1-MCP, the response to the Lovastatin treatment was cultivar dependent. In 'Blanquilla' pear, untreated and Lovastatin-treated fruit exhibited similar levels of ethylene production (Suppl. Fig. 1) but Lovastatin effectively inhibited the accumulation of α-farnesene and disorder incidence (Figs. 2 and 1D). Lovastatin also induced a clear increase of ACO activity (Fig. 2 and Suppl. Fig. 2D) that was not paralleled by higher *PcACO1* gene expression compared to control (Suppl. Fig. 3D). This said, such enhancement of ACO activity is likely transitory since no differences were reported after 4 months of cold-storage (Giné-Bordonaba et al., 2020). Based on our findings, superficial scald development in 'Blanquilla' pears was clearly related to the fruit capacity to produce ethylene and to its regulatory role on *PcAFS1* gene expression during cold storage. However, an improved cold-acclimation capacity associated to 1-MCP treatment (Busatto et al., 2018) or driven by genetic or environmental factors (Marc et al., 2020), is also likely of paramount

importance for the prevention of the disorder. We cannot discard especially the possible involvement of diverse metabolic shifts participating in redox homeostasis and membrane stabilization that may determine the cultivar-specific resistance to superficial scald (Zubini et al., 2007). 1-MCP treatment for instance not only inhibits ethylene production but also consistently leads to enhanced antioxidant enzyme activities (Chiriboga et al., 2013a; Giné-Bordonaba et al., 2020; Vilaplana et al., 2006; Zhi and Dong, 2018; Zhou et al., 2017) and increases the levels of certain cryoprotectants facilitating the stabilization of membranes (Busatto et al., 2018; Giné-Bordonaba et al., 2020). Furthermore, and since ethylene has been shown to be an important repressive regulator of apoplastic H_2O_2 levels in apples (Zermiani et al., 2015), 1-MCP may also promote the expression levels of some genes involved in the ascorbate-glutathione cycle (Zermiani et al., 2015), leading then to higher potential to scavenge ROS and thereby prevent oxidative damage (Giné-Bordonaba et al., 2020; Wang et al., 2018). It is also known that 1-MCP inhibits or delays the gene expressions of glutathione-S-transferases (GSTs) (Karagiannis et al., 2020) and glutathione peroxidases (GPXs) (Wang et al., 2018; Zhou et al., 2017), two enzymes involved in the oxidation of conjugated trienes hydroperoxides to their alcohols (Dixon et al., 2010; Whitaker, 2013). Collectively these results show that the development of superficial scald in 'Blanquilla' pears results from the interaction of several factors and that ethylene, even playing an important role in the synthesis of α-farnesene, did not determine alone the disorder incidence. Future studies investigating the role that ROS scavenging may have in scald control in relation to the initial harvest maturity or to the use of different postharvest storage scenarios is envisaged.

In 'Conference' pears, Lovastatin also reduced the levels of α-farnesene and its oxidation products but did not affect the disorder incidence (Fig. 3 and 1F). These results suggest that α-farnesene is unlikely involved in the development of the disorder observed in this cultivar and further sustained the hypothesis mentioned earlier that the disorder observed in 'Conference' has a completely different etiology than superficial scald. Similar results were also observed by Rizzolo et al. (2015) that, on the basis of the symptom appearance and response to 1-MCP treatment, also suggested that this disorder was not superficial scald.

Finally, the Lovastatin treatment could not control the appearance of superficial scald nor the accumulation of α-farnesene in 'Flor d'Hivern' pears (Figure 1E and 4). Since the Lovastatin effect on *PcAFS1* was fairly similar in all cultivars (Suppl. Fig. 4), it is possible that α-farnesene accumulation in 'Flor d'Hivern', may be partly due to the

synthesis of isopentenyl diphosphate (IPP), a precursor of α-farnesene in the mevalonate pathway, in the plastid via the MEP prior to being transported into the cytoplasm (Eisenreich et al., 2001). Under this scenario, Lovastatin would have little or no effect in the accumulation of α-farnesene in this specific pear cultivar.

5. Conclusions

The results from this study provide detailed information on the distinct processes involved in the cold-induced regulation of scald-like disorders in different pear cultivars. 'Blanquilla' pear showed typical superficial scald symptoms clearly related to the fruit capacity to produce ethylene and to the cold-mediated regulation of *PcAFS1* gene expression. This last link may be considered as a key inducing factor of superficial scald development in this cultivar yet other more complex mechanisms are also likely involved. In contrast to 'Blanquilla', scald control in 'Flor d'Hivern' pears seems to be mainly associated to an improved cold-acclimation process, since this specific cultivar produce undetectable ethylene levels at harvest or upon removal from cold storage. In 'Conference' pear, neither 1-MCP nor Lovastatin inhibited the development of a scald-like disorder and even enhanced it, suggesting the existence of a completely different disorder of unknown etiology that needs to be further investigated.

6. Author's contribution

CL, JGB and VLG conceived and designed the experiment. VLG and ED performed all treatments and storage samplings including quality measurements and sample preparation for biochemical analysis. VLG, NVL and JGB performed the biochemical and molecular analysis. VLG, JGB and CL wrote the manuscript and all remaining authors contributed in improving and revising the final version.

7. Acknowledgments

This work has been supported by the CERCA Programme/ Generalitat de Catalunya and by the Ministerio de Economía y Competitividad / MINECO (grant AGL2017-87923-R). Thanks are also given to the Department d'Empresa i Coneixement de la Generalitat de Catalunya and the European Social Fund 'Investing in your future' for the predoctoral fellowship to VLG (2019FI-B2-00218). We are grateful to Dolors Ubach for her technical support and Agrofresh Inc. for supplying the 1-MCP product.

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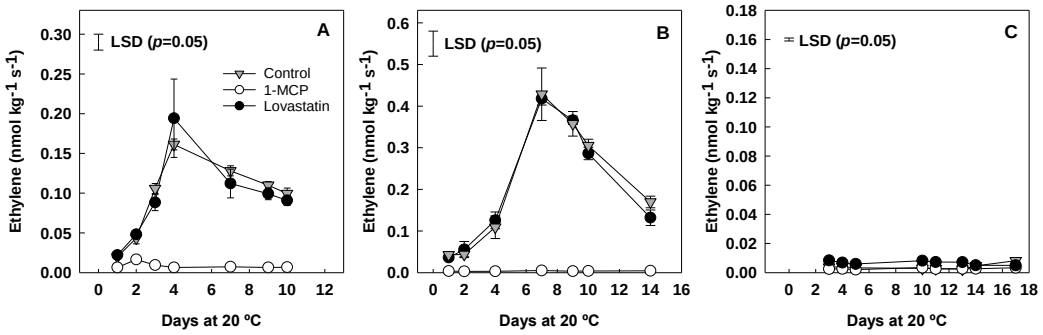
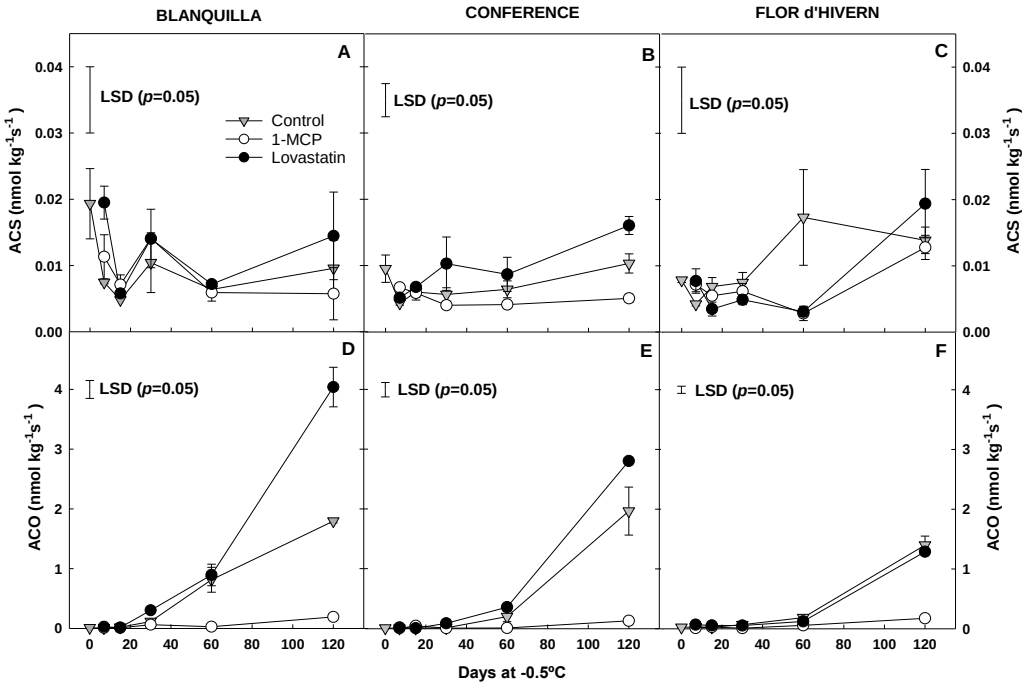
Supplementary Table 1: Primers used for quantitative PCR

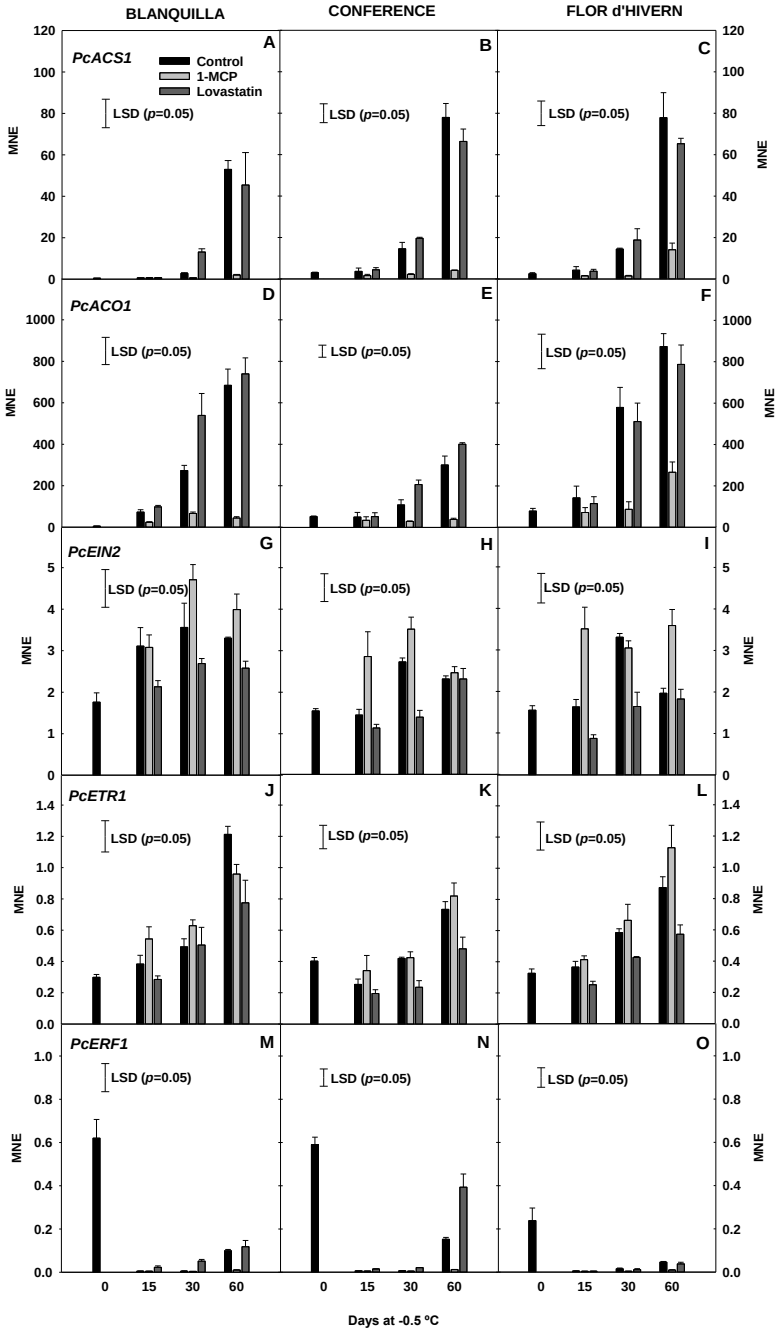
Gene	Annotation	Oligonucleotide sequences	Target gene	Metabolic pathway/Biological function	References
PcACS1	Aminocyclopropane-1-carboxylic acid synthase	F5'-ATGCTGGCTTGTTCTGTTGG-3' R5'-AGGTTCCGTGCAATGACAAG-3'	PCP011500	Ethylene biosynthesis	Busatto et al. (2018)
PcACO1	Aminocyclopropane-1-carboxylic acid oxidase	F5'-AAGGTCAGCAACTACCCTCC-3' R5'-TGTCATCCTGGAAGAGCAGG-3'	PCP011683		Busatto et al. (2018)
PcAFS1	α-farnesene synthase	F5'-GAAAACTAGGCCTCGCGAAC-3' R5'-TTCGATAGCTGCAATGCCGT-3'	PCP028486	α-farnesene biosynthesis	Busatto et al. (2018)
PcHMGR	3-hydroxy-3-methylglutaryl-coenzyme A reductase	F5'-ACGACGGCAAGGACCTTCATG-3' R5'-GCAGGCTGCTTGTGATGCAAG-3'	PCP017787		Giné-Bordonaba et al. (2020)
PcERF1	ethylene response factor 1	F5'-AACATTCGAAACGGCGGAAG-3' R5'-CGAGGACTGAGACGCATTTG-3'	PCP015040	Ethylene response factor	Busatto et al. (2018)
PcETR1	ethylene receptor 1	F5'-AGAACGAGGCGTTGTTGCAC-3' R5'-CCATCATCCCCCATTGCTC-3'	PCP024250	Ethylene signaling	Chiriboga et al. (2013b)
PcEIN2	ethylene insensitive protein 2	F5'-ATCTCTTGTCGAAAGGGCCG-3' R5'-ACGCTTGTAGCGTTTGAGGA-3'	PCP018637.1	Ethylene signaling	This study
Md8283	housekeeping	F5'-CTCGTCGTCTTGTTCCCTGA-3' R5'-GCCTAAGGACAGGTGGTCTATG-3'	PCP030439	housekeeping	Busatto et al. (2019, 2018)



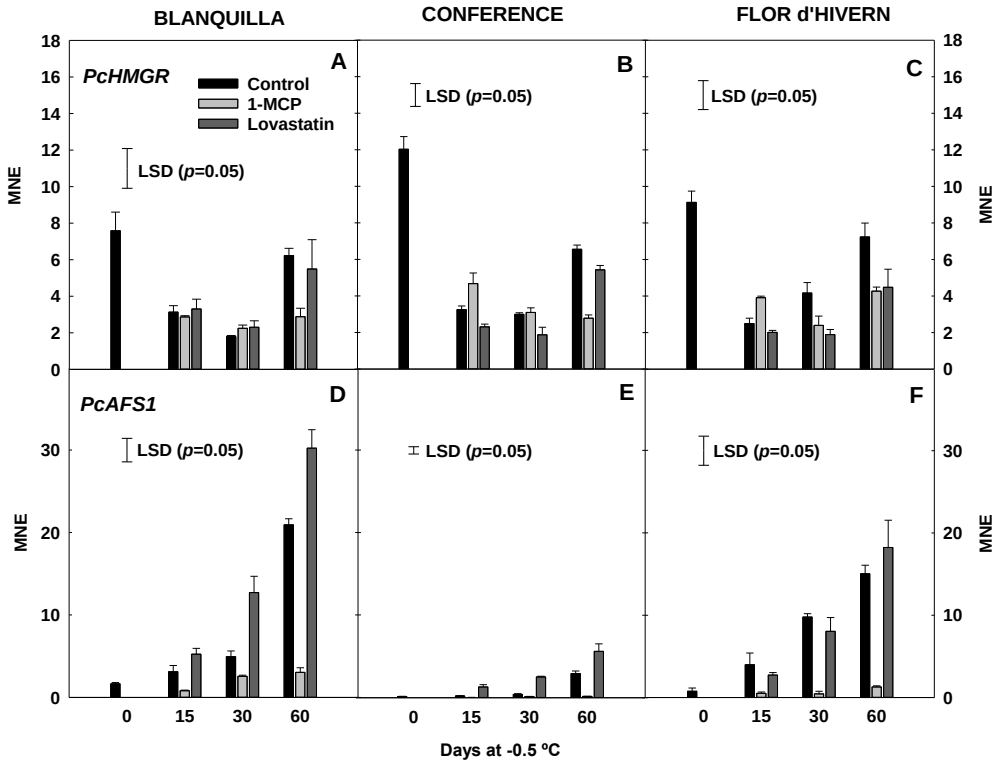
Supplementary Figure 1: Ethylene production upon removal at 20ºC after 60 d of cold storage in 'Blanquilla' (A), 'Conference' (B) and 'Flor d'Hivern' (C). Error bars represent the standard error of the means (n=3). Single error bar depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.



Supplementary Figure 2: ACC synthase (A, B and C) and ACC oxidase (D, E and F) activities in the three cultivars studied along cold storage. Error bars represent the standard error of the means (n=3). Single error bar depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.



Supplementary Figure 3: *PcACS1* (A, B and C), *PcACO1* (D, E and F), *PcEIN2* (G, H and I), *PcETR1* (J, K and L) and *PcERF1* (M, N and O) gene expressions in the three cultivars studied along cold storage. Error bars represent the standard error of the means (n=3). Single error bar depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.



Supplementary Figure 4: *PcHMGR* (A, B and C) and *PcAFS1* (D, E and F) gene expressions in the three cultivars studied along cold storage. Error bars represent the standard error of the means (n=3). Single error bar depicts the LSD value (p=0.05) for the interaction treatment*sampling of cold storage.

DISCUSIÓN GLOBAL

Como ya se comentó en profundidad en la introducción, el escaldado superficial es de las alteraciones fisiológicas más importantes en pera y, sin embargo, la mayoría de las investigaciones realizadas para entender esta alteración se han llevado a cabo en manzana. El modelo en manzana está bastante bien establecido y cabría esperar que se pudiese extrapolar sin ningún problema a otra pomácea como es la pera, pero no es así. El escaldado en pera requiere ser estudiado aparte, tomando por guía el modelo de manzana, pero teniendo en cuenta que no se van a cumplir todas las premisas. El mejor ejemplo es el estado de madurez, ya que en manzanas se sabe que la fruta más inmadura es más susceptible al escaldado (Emongor et al., 1994), mientras que en la pera, por regla general, ocurre lo contrario, observándose una incidencia mayor cuanto más madura se cosecha la fruta (Calvo et al., 2015). Quizás este es un hecho aislado o quizás esto nos da a entender que peras y manzanas no son especies tan parecidas como parecen…

Es cierto que el escaldado superficial aparece durante la vida útil del fruto después de haber pasado un largo periodo de almacenamiento en frío, y es por esto que la mayoría de las investigaciones realizadas están orientadas a estudiar esta fisiopatía después de la conservación y evaluando la respuesta a diferentes tratamientos, condiciones de conservación, fechas de cosecha, etc. (Calvo et al., 2018, 2015; Ekman et al., 2004; Larrigaudière et al., 2019). Sin embargo, en esta tesis se quiere resaltar la importancia que tiene la fase precosecha, y sobre todo cómo los cambios fisiológicos que ocurren en esta fase pueden determinar el posterior comportamiento de un fruto. Y, ¿Por qué? Pues porque para tratar de solucionar un problema hay que ir a la base de él y definir si la acumulación o ausencia de cierto metabolito puede influir en el posterior comportamiento del fruto en frente a diferentes situaciones de estrés, como puede ser la conservación frigorífica. Es por ello que, de los cinco capítulos que consta esta tesis, 3 de ellos se han dedicado a estudiar la maduración de diferentes variedades de pera y los otros, a estudiar el desorden fisiológico en sí.

1. La implicación del etileno en los primeros estadios del crecimiento y la importancia del ABA, IAA y GA_1 durante el desarrollo de "Blanquilla" y "Conference"

En el Capítulo 1 se abordó toda la fase de crecimiento y desarrollo del fruto para evaluar el comportamiento de dos variedades de pera con diferentes necesidades de frío para conseguir una maduración normal tras ser cosechada. "Blanquilla" es una típica pera de verano capaz de madurar y producir etileno nada más ser cosechada,

sin necesidad de recibir ningún tratamiento con frío. Por otro lado, "Conference" se puede considerar una pera intermedia entre verano e invierno puesto que sí necesita un periodo de almacenamiento en frío pero corto, de tan sólo 2 semanas.

Respecto al ratio de crecimiento, no se observaron muchas diferencias puesto que ambas variedades mostraron dos fases bien diferenciadas, al igual que se ha descrito en pera japonesa (Zhang et al., 2008a). Sin embargo, los resultados obtenidos no concordaron con las 3 fases que se comentaron en la introducción. Esta diferencia se puede explicar de forma sencilla considerando que la pera se suele cosechar en un estadio preclimatérico, en un punto en el que la fruta ya ha alcanzado la madurez fisiológica, por lo que la fase III (donde se producen los diferentes cambios en firmeza, color, aroma, etc.) sucede en gran parte durante su conservación. Antes de continuar es importante aclarar algunos conceptos. En castellano únicamente se suele utilizar el término "maduración" para referirnos al proceso evolutivo de la fruta y a los diferentes cambios que se producen en ella hasta que se convierte en un producto atractivo para el consumo; sin embargo, en inglés se utilizan dos palabras: *maturation* y *ripening*. El término *maturation* hace referencia a los cambios que se producen durante el desarrollo en el árbol y que termina cuando los frutos alcanzan la madurez fisiológica, es decir, cuando el fruto es capaz de madurar aunque se separe de la planta madre. Por otro lado tenemos el término *ripening*, el cual engloba todos los procesos que tienen lugar antes de la senescencia del fruto y que incluye cambios en la textura, el color, aroma, etc. (Kader, 1999).

Centrándonos en el etileno, los resultados mostraron que, a pesar de ser la hormona por excelencia durante la maduración (*ripening*) (Pech et al., 2012), esta hormona también desarrolla un papel clave en los primeros estadios del desarrollo del fruto. El papel del etileno durante el crecimiento de la planta está bien descrito (Vandenbussche y van der Straeten, 2012), pero pocos estudios hay sobre su papel durante el desarrollo del fruto probablemente por el hecho de que su función puede variar dependiendo de la planta y del balance con otras hormonas (Small y Degenhardt, 2018). La producción elevada de etileno en los primeros estadios del desarrollo podría deberse a que esté involucrado en el proceso de transición floral, tal y como se ha demostrado en mutantes de Arabidopsis (Ogawara et al., 2003). El etileno es también, un regulador de la división celular e inhibidor de la expansión celular (Schaller, 2012), de ahí que su producción decrezca hasta niveles basales cuando comienza la fase de crecimiento exponencial del fruto.

Dejando un poco de lado el etileno, lo realmente interesante y novedoso de esta parte del trabajo es la implicación que tienen el resto de hormonas, a las cuales no se les suele dar mucha importancia en los frutos climatéricos pero que, sin embargo, parecen estar involucradas en algunos procesos clave. A pesar de que, en mayor o menor medida, las cinco hormonas estudiadas son importantes, aquí en la discusión destacaremos tres de ellas: el ácido abscísico (ABA), el ácido 3-indolacético (IAA) y la giberelina 1 (GA_1).

Empezando por el ABA podemos decir que, desde hace años, se sabe que es la hormona por excelencia en la maduración de frutos no climatéricos (Jia et al., 2011; Setha, 2012), y que se ha relacionado positivamente con la acumulación de antocianinas y sólidos solubles en cerezas (Tijero et al., 2016; Wang et al., 2015) y con la pérdida de firmeza en uva (Castellarin et al., 2016); sin embargo, poco a poco se le ha ido dando importancia al ABA en frutos climatéricos, y se ha visto que su contenido aumenta progresivamente desde estadios inmaduros hasta la fecha de cosecha en manzana (Setha et al., 2004; Vendrell y Buesa, 1989) y tomate (Zhang et al., 2009). *A priori* parece que es justo lo contrario a lo que ocurre en "Blanquilla" y "Conference", pero si nos fijamos en la fase exponencial del fruto, se puede observar una cierta tendencia a la acumulación de ABA sobre todo en pera "Blanquilla". La diferencia en el contenido de ABA en cosecha entre ambas variedades podría estar relacionada con la capacidad de producir etileno, ya que el ABA promueve la síntesis de etileno a través de la inducción de la ACS y la ACO y la acumulación del precursor ACC (Mou et al., 2016; Zaharah et al., 2013). Además de afectar a la síntesis de etileno, el ABA también parece estar involucrado en la acumulación de azúcares, sobre todo la sacarosa (Setha, 2012). Esta premisa se vio reflejada en nuestros resultados con pera "Blanquilla", ya que los niveles de sacarosa se mantienen estables hasta que la concentración de ABA alcanza su mínimo, pero aumentan después de manera paralela.

Dentro de las auxinas, el IAA es sin duda la más abundante en plantas (Santner et al., 2009). A diferencia de lo que se observa en frutos no climatéricos, donde se ha visto que el IAA parece afectar solo a los primeros estadios del fruto (Estrada-Johnson et al., 2017), en frutos climatéricos existen diferentes estudios que recalcan su importancia durante el crecimiento del fruto, sobre todo por su acción estimuladora de la producción de etileno tal y como se ha visto en melocotón (Trainotti et al., 2007), manzana (Yue et al., 2020) y tomate (Quinet et al., 2019). Quizás lo más remarcable de las auxinas en general es la interacción que tiene con el resto de hormonas, que

parecen cooperar entre ellas para conseguir un correcto desarrollo. Por ejemplo, una acción equilibrada entre auxinas, giberelinas y citoquininas hacen posible que el cuajado del fruto (*fruit set*) se desarrolle de forma correcta, ya que la acción individualizada de cada una de ellas no lo lograría (Mariotti et al., 2011). También las auxinas, junto con las citoquininas, son clave en el desarrollo del fruto (Kumar et al., 2014), y algunos autores han demostrado que las auxinas son las encargadas principales de regular el tamaño del fruto en manzana (Devoghalaere et al., 2012). Durante la maduración (*maturation*) también son clave ya que se ha visto que en mango es necesaria una reducción en el contenido de auxinas antes de que comience el proceso de *ripening* (Zaharah et al., 2012). Por último, pero no por ello menos importante, varios estudios demuestran que existe un fuerte *cross-talk* entre etileno y auxinas durante el periodo de maduración (*ripening*). Esta interacción afecta principalmente el nivel de biosíntesis de etileno, ya que las auxinas promueven la expresión de los genes de la ACC sintasa, como se ha demostrado en tomate (Abel y Theologis, 1996). Investigaciones posteriores refuerzan este *cross-talk* resaltando la interacción a nivel de expresión y regulación génica (Busatto et al., 2017; Li et al., 2016). En nuestros resultados, "Blanquilla" mostró una tendencia ligeramente ascendente en el contenido de IAA mientras que en "Conference" los valores descienden conforme el fruto madura, observándose en cosecha una gran diferencia en el contenido de esta hormona entre ambas variedades (Fig. 1). Este comportamiento y, en particular, los elevados valores de IAA en el momento de la cosecha de "Blanquilla" podrían explicar la capacidad de esta variedad de producir etileno nada más ser cosechada.

Las GAs son un gran grupo de hormonas que también participan en diferentes procesos fisiológicos, desde el desarrollo de la semilla hasta la maduración del fruto (Santner et al., 2009). La acción de esta hormona se asocia principalmente con los primeros estadios del fruto, ya que promueve la expansión celular y, junto con auxinas y citoquininas promueven el cuajado del fruto (Kumar et al., 2014; Ruan et al., 2012; Zhang et al., 2008b). De hecho, algunos artículos han demostrado que el IAA promueve la síntesis de giberelinas en diferentes especies, como Arabidopsis (Frigerio et al., 2006) y tomate (Serrani et al., 2008). También existe una fuerte relación entre las giberelinas y el etileno, ya que se ha visto que el etileno puede afectar a la síntesis de giberelinas (Ross et al., 2016) pero también se ha demostrado el efecto inverso, donde las giberelinas inhiben la síntesis de etileno (De Grauwe et al., 2008) y la maduración del fruto (Dostal y Leopold, 1967). Un estudio reciente ha demostrado que las giberelinas regulan la maduración en tomate mediante una ralentización de la

conversión del sistema 1 al sistema 2 en la producción de etileno, de ahí la incapacidad de los frutos de producir el pico climatérico y, por lo tanto, madurar (Li et al., 2019). En nuestros resultados se observó que los niveles de GA_1 en "Blanquilla" descienden conforme avanza la maduración, mientras que en "Conference" el contenido de esta hormona aumentó considerablemente, sobre todo en los últimos estadios (Fig. 1). Los altos niveles de GA_1 observados en "Conference" en el momento de cosecha, y en comparación con los mostrados por "Blanquilla", podrían explicar la incapacidad de esta variedad de producir etileno en cosecha. Además, en algunas especies se ha visto que el contenido en giberelinas disminuye con las temperaturas bajas (Pinthus et al., 1989; Reid et al., 1974), por lo que esto podría explicar también en parte la necesidad de "Conference" de ser sometida a un periodo en frío para poder madurar.

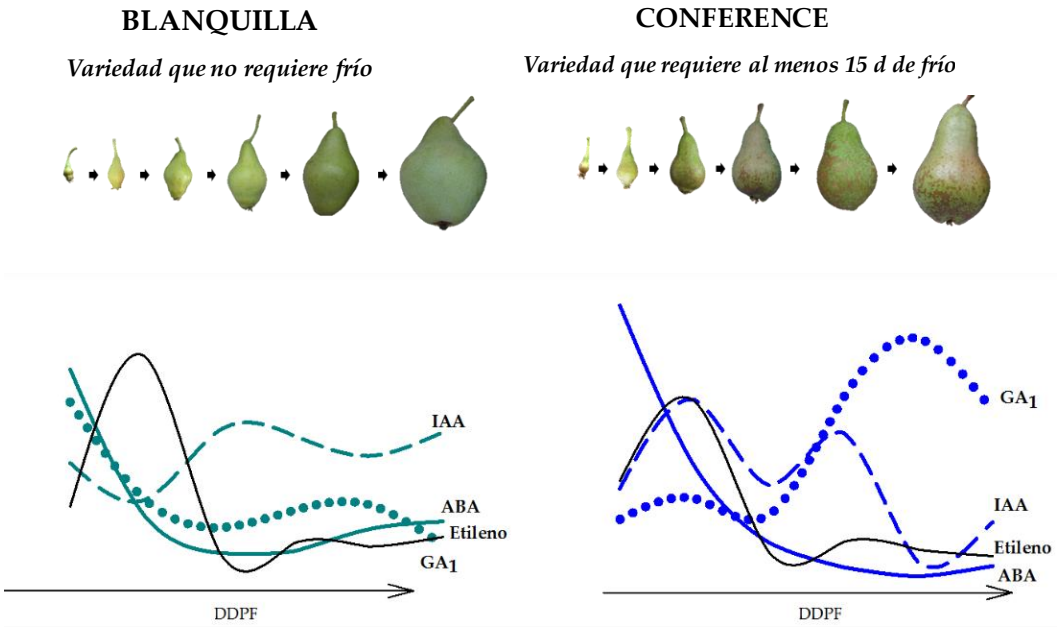


Figura 1. Esquema de la evolución del etileno, ABA, IAA y GA_1 en las dos variedades estudiadas. DDPF = Días Después de Plena Floración (Las concentraciones de hormonas no están representadas a escala).

2. Diferentes patrones de maduración en árbol y poscosecha en pera "Blanquilla", "Conference" y "Flor d'Hivern"

2.1. La maduración en peras de verano está mediada por el etileno, la sacarosa y el estrés oxidativo

Teniendo en cuenta el plan de trabajo y los resultados obtenidos en el Capítulo 1, en el Capítulo 2 se decidió continuar con el estudio de la evolución del fruto, comparando los patrones de maduración en árbol y fuera de árbol (poscosecha) de la variedad "Blanquilla".

En general, la pera se considera un fruto incapaz de madurar en el árbol, al igual que el aguacate o el mango, y a diferencia de otros frutos climatéricos como la manzana y el tomate (Murayama et al., 2015). Diferentes estudios han demostrado la capacidad que tienen algunas peras como "d'Anjou" o "Bartlett" para perder firmeza mientras están madurando en el árbol, si bien los valores de firmeza nunca llegan a ser los adecuados para su consumo (unos 20 N aproximadamente) (Bai et al., 2009; Murayama et al., 1998; Raffo et al., 2012; Sugar y Einhorn, 2011). El porqué de esta inhibición en el árbol era una incógnita, hasta que Murayama et al. (2015) propusieron dos teorías: I) el etileno no se produce hasta el día óptimo de cosecha y, una vez que el fruto empieza a producir etileno, este promueve la abscisión antes de que se complete el proceso de maduración y II) el continuo transporte de ciertos metabolitos de la planta madre al fruto puede inhibir o retrasar la maduración (lo que haría alusión al conocido *tree factor*).

A diferencia de los resultados obtenidos en otras variedades (Murayama et al., 2006, 1998; Raffo et al., 2012; Sugar y Einhorn, 2011), nuestros estudios realizados en pera "Blanquilla" demostraron que esta variedad es capaz de producir etileno y perder firmeza durante la maduración en el árbol. La capacidad de madurar en el árbol se relacionó en este caso con el metabolismo del etileno y en concreto con las activaciones de la ACS sintasa y ACC oxidasa en el árbol. En cambio, la pérdida de firmeza en poscosecha (*off-tree*) parece estar inducida por un proceso oxidativo, que resulta en la acumulación del malondialdehído (MDA). El MDA es una molécula pequeña (3 átomos de carbono y 2 grupos aldehído) pero de elevado interés biológico, ya que es un producto secundario de la oxidación de los ácidos grasos poliinsaturados (Hodges et al., 1999) que se suele utilizar como marcador de peroxidación lipídica de las membranas celulares (Morales y Munné-Bosch, 2019). Un aumento en el contenido

de MDA se ha observado durante el desarrollo de cereza (Giné-Bordonaba et al., 2017) y tomate (Mondal et al., 2004) y también durante el periodo de ablandamiento de plátano (Yang et al., 2008) y albaricoque (Wu et al., 2015) durante la conservación a temperatura ambiente. Este aumento en el contenido de MDA puede deberse al mismo proceso de senescencia, tal y como se ha relacionado en hoja de Arabidopsis (Dhindsa et al., 1981) y en melón (Lacan y Baccou, 1998). Además, algunos autores resaltan la importancia del MDA ya no sólo como una molécula reactiva que puede causar un daño celular, sino también una molécula capaz de activar la expresión génica y actuar como un modulador de la señalización celular mediante la activación de las enzimas aldehído deshidrogenasas (Farmer y Davoine, 2007; Tagnon y Simeon, 2017; Weber et al., 2004), las cuales son las encargadas de eliminar de las células los aldehídos tóxicos procedentes de la degradación de las membranas celulares (Brocker et al., 2013).

Volviendo a la maduración de los frutos en el árbol, hay que destacar la importancia que pueden tener también los azúcares, y en concreto la sacarosa, ya que en este capítulo se propuso la hipótesis de que la acumulación de este disacárido podría ser necesaria para que el fruto produjese etileno mientras estuviese unido a la planta. Los azúcares han sido considerados durante mucho tiempo como simples sustratos respiratorios, intermediarios metabólicos y componentes estructurales o de almacenamiento a pesar de que cada vez los estudios profundizan más en su interés como moléculas de señalización (Sheen et al., 1999; Smeekens, 2000). El no haber considerado los azúcares como moléculas de señalización podría deberse a las altas concentraciones en las que se encuentran en la planta, ya que las hormonas están presentes en el rango nano o micromolar, mientras que los azúcares en milimolar (Sheen et al., 1999; Smeekens, 2000). Sin embargo, su importancia es obvia ya que los azúcares están involucrados en muchos procesos vitales para la planta. Por ejemplo, se ha visto que la sacarosa induce la expresión de ciclinas de tipo D (las cuales son reguladores del ciclo celular) en cultivos celulares de Arabidopsis (Riou-Khamlichi et al., 2000). La sacarosa también afecta a la transición floral (Ohto et al., 2001) y su metabolismo está relacionado con la adquisición de tolerancia al frío (Klotke et al., 2004; Wanner y Junttila, 1999). También se ha observado que un aumento en la concentración de azúcares puede inducir la expresión de proteínas relacionadas con la patógenesis (Roitsch, 1999) e inducir la acumulación de proteínas antifúngicas durante la maduración de la uva (Salzman et al., 1998). La senescencia también parece estar regulada por el nivel de azúcares de la planta, ya que algunos estudios han demostrado que un alto contenido en azúcares puede inducir senescencia en hojas de

Arabidopsis (Pourtau et al., 2006; Wingler et al., 2012). En la revisión de Gibson (2005) se puede encontrar un resumen detallado de los procesos que están regulados por azúcares.

Como podemos comprobar, se le da mucha importancia a la sacarosa, debido probablemente a que es el principal azúcar translocado y el más utilizado en la mayoría de los estudios (Sheen et al., 1999). Sin embargo, es difícil establecer una función directa de la sacarosa ya que esta es transportada e hidrolizada en glucosa y fructosa, por lo que en ocasiones es difícil saber si un determinado efecto es debido al disacárido o las hexosas (Wind et al., 2010). Además de la importancia de los azúcares por sí solos, también es remarcable la interacción existente con las hormonas. Varios estudios muestran que existe un *cross-talk* bidireccional entre azúcares y ABA ya que, por un lado, el ABA es capaz de potenciar la expresión de genes de la biosíntesis del almidón (Rook et al., 2002) y por el otro, la aplicación de glucosa potencia la expresión de genes de biosíntesis de ABA (Cheng et al., 2002). Además, también se ha visto que el etileno puede actuar como un antagonista en la ruta de señalización de la glucosa, probablemente mediante la inhibición de la biosíntesis de ABA (León y Sheen, 2003). Algunos estudios también han mostrado el *cross-talk* existente entre azúcares y auxinas a nivel de las rutas de señalización (Leclere et al., 2010; Sagar et al., 2013).

Lo más destacable de nuestros resultados es la capacidad que tiene la variedad "Blanquilla" de madurar en el árbol, llegando a alcanzar valores de firmeza aptos para el consumo y similares a los obtenidos durante la maduración poscosecha. Esta capacidad de madurar en el árbol podría estar relacionada con la acumulación de sacarosa, la cual es capaz de inducir la producción de etileno (potenciando la actividad ACO) y, por lo tanto, promover la pérdida de firmeza (Fig. 2). Además, y aunque en este capítulo no se analizó el perfil hormonal, la capacidad de madurar en el árbol podría estar también relacionada con un alto contenido en IAA y ABA, los cuales serían suministrados por la planta, promoviendo así la síntesis de etileno. Por otro lado, la maduración poscosecha pareció no estar relacionada con un aumento de la sacarosa, ya que es muy probable que ésta sea hidrolizada a glucosa y fructosa una vez ha sido separada del árbol. La maduración poscosecha pareció estar mediada por un proceso oxidativo, el cual promueve la producción de etileno (potenciando en este caso la actividad de la enzima ACS) y favorece la pérdida de firmeza (Fig. 2).

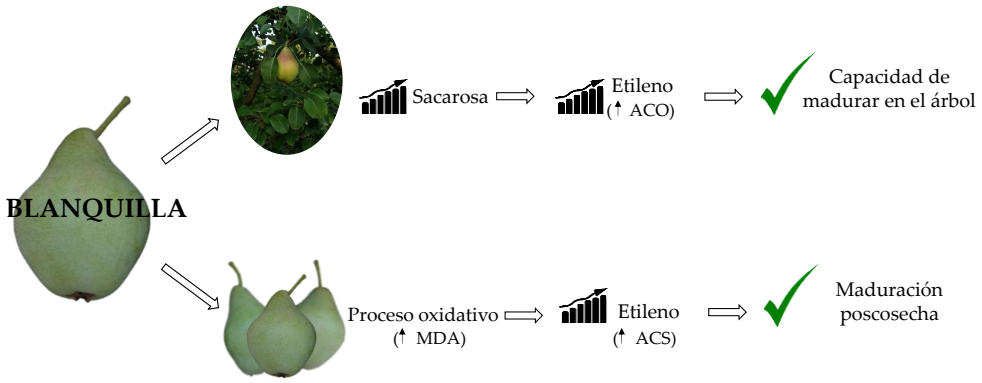


Figura 2. Esquema de los patrones de maduración en árbol y fuera de árbol en pera "Blanquilla".

2.2. La incapacidad de madurar en árbol de "Conference" y el inusual metabolismo del ACC de "Flor d'Hivern"

Teniendo en cuenta los resultados obtenidos en pera "Blanquilla", nos preguntamos: ¿Seguirán el mismo patrón de maduración otras peras de verano? ¿Y las peras de invierno? Y de ahí que decidiésemos hacer un estudio muy similar con las variedades "Conference" y "Flor d'Hivern" (Capítulo 3).

Los resultados obtenidos en el Capítulo 3 nos mostraron que las similitudes con el patrón de maduración de "Blanquilla" eran más bien escasas. No obstante, sí que se observaron similitudes entre "Conference" y "Blanquilla" en algunos aspectos de la maduración poscosecha como son la pérdida de firmeza, el aumento del índice de almidón y la disminución del índice de diferencia de absorbancia de la clorofila (I_{AD}). Para la discusión, nos centraremos primero en la variedad "Conference", la cual como se ha dicho antes requiere un corto periodo de almacenamiento en frío para madurar. Se ha visto que las bajas temperaturas disminuyen el contenido en giberelinas (Pinthus et al., 1989; Reid et al., 1974) y, como ya se discutió en el primer apartado de la discusión, el alto contenido en giberelinas (y en concreto GA_1) observado en "Conference" podría estar inhibiendo la producción de etileno. Es seguramente por este motivo que la producción de etileno en "Conference" fue extremadamente baja durante la maduración a 20 °C, al igual que se ve en otras variedades típicas de invierno, como "Comice" (López et al., 2001; Ma y Chen, 2003) o "d'Anjou" (Gerasopoulos y Richardson, 1997), las cuales no muestran producción de etileno hasta después de, como mínimo, 30 y 60 días de almacenamiento en frío, respectivamente (Sugar, 2011).

Dos cosas nos pueden llamar la atención en cuanto al comportamiento de "Conference" tras la cosecha. La primera es que, a pesar de no producir etileno, sí se observaron grandes pérdidas de firmeza y de IAD, a diferencia del estudio de Gerasopoulos y Richardson (1997) con la variedad de invierno "d'Anjou". De hecho, en un gran número de estudios realizados en otras variedades de pera y también en tomate, se mostró que la pérdida de firmeza suele estar asociada a una elevada producción de etileno (Fuggate et al., 2010; Murayama et al., 2015, 2006). En este caso, la falta de producción de etileno después de cosecha podría deberse a una cosecha en estadio preclimatérico (Chiriboga et al., 2011).

El segundo aspecto que llamó bastante la atención es que, aunque no produjese etileno, tanto las actividades enzimáticas de ACS y ACO como el contenido en ACC eran bastante elevados. Normalmente la actividad de la ACS se considera el paso limitante en la producción de etileno (Yang y Hoffman, 1984) aunque otros artículos consideran que podría ser la ACO cuando el fruto se encuentra en la fase de *ripening* (Vanderstraeten y van Der Straeten, 2017); sin embargo, ninguna de estas premisas se cumplían en "Conference" ya que ambas enzimas mostraron una actividad elevada que no se tradujo en una mayor producción de etileno. Entonces, ¿Por qué la ACO no es capaz de oxidar el ACC existente y producir etileno? Lo primero que podríamos pensar es que la actividad de la ACO es baja e insuficiente para transformar el ACC en etileno, pero esto queda descartado si comparamos con el rango de valores obtenido en "Blanquilla" (Capítulo 2). Se podría pensar también que, aunque se observó un gran incremento en el contenido de ACC, esta concentración es insuficiente para que se produzca la conversión a etileno por el hecho que la ACO se encuentra en un estado de menor sensibilidad (más próximo a un estado en el sistema 1). Esta teoría va en línea con la hipótesis lanzada anteriormente en la que se asumía que "Conference" se cosechó con una menor madurez fisiológica. El tercer y último motivo, y quizás el más probable, es que el ACC formado no se esté utilizando como sustrato de la ACO sino para otros fines. El ACC se ha considerado de siempre como el precursor directo del etileno en plantas superiores (Vanderstraeten y van Der Straeten, 2017). Sin embargo, algunos estudios han demostrado que también podría tener otras funciones vitales para la planta. Por ejemplo, un estudio llevado a cabo por Tsuchisaka et al. (2009) mostró que en mutantes de *Arabidopsis thaliana* con todas las isoformas del gen de la ACS suprimidas, se producía la muerte del embrión y, sin embargo, mutantes con algunos de los componentes de la ruta de señalización del etileno suprimida (p. ej: mutantes *ein2*, insensibles al etileno) eran totalmente viables. Esto indicaría que una planta puede sobrevivir teniendo alterada la ruta de

señalización del etileno, pero no sin la síntesis de ACC. También se ha realizado otro estudio en tomate y se ha visto que, en situaciones de exceso de agua en el medio, la síntesis de ACC aumenta en las raíces y se transporta hasta las ramas, donde se propicia la síntesis de etileno y epinastia (curvatura de las hojas hacia abajo en situaciones de estrés). Cuando se retira el exceso de agua y las plantas dejan de estar bajo estrés abiótico, el flujo de ACC y la síntesis de etileno disminuyen (Bradford y Yang, 1980). Resultados similares también se han observado en cítricos con déficit de agua que aumentan la síntesis de ACC en raíces una vez han sido rehidratados. El ACC viaja hasta las ramas donde se produce la conversión a etileno, y éste es quien provoca la abscisión de las hojas (Tudela y Primo-Millo, 1992).

Aunque en estos dos últimos casos es el etileno quien tiene la función de provocar abscisión o epinastia, el ACC además de servir como precursor, también actúa como una señal. A pesar de estos ejemplos, el ACC no sólo tiene función bajo situaciones de estrés, ya que también hay varios estudios que demuestran su participación en la senescencia y polinización de flores (Vanderstraeten y van Der Straeten, 2017) y también en el desarrollo de los estomas (Yin et al., 2019). Además, una función del ACC totalmente independiente del etileno es la atracción de rizobacterias que promueven el crecimiento de la planta. En este caso las plantas liberan a la rizosfera el ACC, el cual es utilizado por algunas bacterias como fuente de carbono y nitrógeno gracias a la acción de las ACC desaminasas (Glick et al., 1998); al reducirse el *pool* de ACC, se reduce también la síntesis de etileno favoreciéndose la elongación de las raíces.

Todos estos ejemplos, aunque hablen de la planta en general y no del fruto, dan a entender que el ACC no es únicamente sintetizado como precursor del etileno, sino que tiene otras múltiples funciones… Quizás algunas de ellas todavía desconocidas.

Dejando de lado las otras funciones del ACC, este también puede conjugarse y formar unos derivados. La formación de estos derivados permite la regulación del *pool* existente de ACC, pero no se conoce mucho sobre sus funciones (Vanderstraeten y van Der Straeten, 2017). La primera forma conjugada del ACC que se descubrió fue el malonil-ACC (MACC) (Hoffman et al., 1982), el cual es formado gracias a la acción de la ACC malonil-transferasa. Años más tarde se vio que el tratamiento con etileno promueve la actividad de la malonil-transferasa, aumentándose la formación de MACC y reduciéndose por tanto la oxidación de ACC a etileno (Liu et al., 1985). Por otro lado, el MACC en algunas situaciones puede hidrolizarse para formar ACC (Jiao

et al., 1986), demostrando así que la función de este proceso de conjugación podría ser la de controlar los niveles disponibles de ACC. Otro derivado del ACC es el γ-glutamil-ACC (GACC), formado por la enzima γ-glutamiltranspeptidasa (Martin et al., 1995), pero poco se sabe de él y sus funciones. El tercer conjugado es el jasmonil-ACC (JA-ACC), formado gracias a la enzima jasmonato-amino sintetasa (comúnmente conocida como JAR1). La formación de JA-ACC podría ser una clara evidencia del *cross-talk* existente entre las hormonas jasmonato y etileno (Staswick y Tiryaki, 2004).

Por lo tanto, todos estos motivos podrían hacer que el ACC no estuviese disponible para la síntesis de etileno y de ahí que, a pesar de tener toda la maquinaria del metabolismo activada, "Conference" no sea capaz de producir etileno. Este hecho también pone de manifiesto la existencia de un proceso de pérdida de firmeza etileno-independiente, ya que, a pesar de no observarse producción de etileno, los frutos madurados en cámara a 20 °C (*off-tree)* se ablandaron rápidamente después de cosecha. Diferentes estudios en mutantes con la ruta de síntesis de etileno suprimida han demostrado la existencia de eventos de la maduración que son etileno-independientes (Pech et al., 2008). Uno de los eventos más estudiados, quizás por ser de los parámetros más importantes en la maduración de un fruto, es la pérdida de firmeza. Pech et al. (1999) demostraron que, en melón, hay enzimas degradadoras de la pared celular que son etileno-dependientes (endo-poligalacturonasa y galactanasa) pero que hay otras etileno-independientes (pectinmetilesterasa y exo-poligalacturonasa). Estas últimas podrían ser las responsables de la pérdida de firmeza en poscosecha (*off-tree)* observada en la variedad "Conference".

Por otro lado, los frutos madurados en el árbol sí que parecieron mostrar un comportamiento etileno-dependiente, ya que no producían etileno, pero tampoco se observó una pérdida de firmeza y un cambio del color remarcables. En este caso cobra real importancia la teoría del *tree factor* (Abeles et al., 1992), la cual habla de la existencia de una molécula, aún de naturaleza desconocida, que es suministrada por la planta e impide que el fruto madure mientras esté unido a ella. Los estudios de esta molécula empezaron a realizarse en aguacate (Gazit y Blumenfeld, 1970; Tingwa y Young, 1975), pero poco a poco se han ido realizando estudios en manzana (Lin y Walsh, 2008), caqui (Sun et al., 2013) y también en pera (Murayama et al., 2015). Todos ellos muestran que los frutos maduran de manera más rápida una vez han sido cosechados, excepto el aguacate que no es que madure más lentamente sino que si no es retirado de la planta madre no madura.

En base a nuestros resultados se pueden proponer diferentes moléculas como candidatas a *tree factor* (Fig. 3). Por un lado está la giberelina 1, la cual hemos visto que probablemente sea la responsable de la incapacidad de producir etileno en cosecha de la variedad "Conference". En este caso, la GA_1 estaría siendo suministrada por la planta y, al separar la fruta de la planta madre, el suministro de giberelinas cesa. En el caso de que el contenido de éstas fuese elevado, sería necesario un periodo de frío para que disminuyesen los niveles de estas hormonas y se promoviese así la producción de etileno. Por otro lado, también podríamos hablar de la sacarosa como *tree factor*; aunque en este caso no estaríamos ante una molécula inhibidora sino una que promueve la maduración. Los resultados obtenidos en el Capítulo 2 demostraron que la capacidad de producir etileno en los frutos madurados en el árbol estaba relacionada con un aumento en la concentración de sacarosa. Comparando los niveles de este azúcar en "Blanquilla" con los obtenidos en "Conference" vemos que el contenido es prácticamente el doble en la primera variedad, por lo que podríamos decir que se necesita una cantidad umbral de sacarosa para poder inducir la producción de etileno y, por lo tanto, promover la maduración de los frutos en el árbol. Por último, también se podría proponer como *tree factor* a esa molécula (aún por definir) que hace que se mantengan altos los niveles de H_2O_2 y bajos los de MDA mientras el fruto está unido a la planta, y que probablemente hace que los frutos sean más resistentes al daño oxidativo; tal y como se ha visto en "Conference" y "Flor d'Hivern" (Capítulo 3).

Una vez ya hemos hablado de la variedad "Conference", ahora nos centraremos en "Flor d'Hivern". Como ya se comentó en la introducción, ésta es una variedad local y existen muy pocos estudios sobre la fisiología de su maduración y almacenamiento. Respecto a los parámetros de calidad estudiados, el comportamiento de "Flor d'Hivern" se podría asemejar en cierta parte al observado en "Conference", observándose un ablandamiento y un descenso en el valor del I_{AD} de los frutos madurados fuera de árbol, mientras que en los frutos madurados en el árbol estos parámetros se mantienen estables. También se observó un aumento en el índice de almidón en ambas condiciones, con la diferencia de que los valores iniciales de "Flor d'Hivern" eran mucho más altos que los de "Conference". Otra similitud con "Conference" es que los cambios cualitativos en poscosecha no se pudieron asociar a la producción de etileno, ya que los niveles de producción observados fueron incluso más bajos que los observados en "Conference". Una de las diferencias entre las dos variedades es que, como ya se comentó en el Capítulo 3, los cambios observados en "Conference" en los frutos madurados tras la cosecha se podían asociar a un proceso

oxidativo de la misma manera que en "Blanquilla" (Capítulo 2). En el caso de "Flor d'Hivern", la maduración de los frutos poscosecha no se pudo asociar a la producción de etileno, pero tampoco a un proceso oxidativo ya que el MDA mostró unos valores similares entre ambas condiciones de maduración. Por lo tanto, futuros estudios son necesarios para entender qué procesos fisiológicos o bioquímicos son los responsables de la lenta maduración poscosecha observada en la variedad "Flor d'Hivern".

Lo más destacable de la variedad "Flor d'Hivern" es sin duda su metabolismo del etileno, del cual sorprende la alta actividad de la enzima ACC oxidasa en los frutos madurados en poscosecha (*off-tree)*. En este caso, sí podemos decir que el paso limitante en la producción de etileno es la ACC sintasa, ya que probablemente no esté sintetizando suficiente ACC que pueda ser oxidado a etileno y de ahí que, aunque la actividad de la ACO sea elevada, no se traduzca en una concentración de etileno considerable. Por otro lado, también podría ser que el poco ACC sintetizado se esté utilizando para otras funciones, como ya se comentó con "Conference" (Polko y Kieber, 2019; Vanderstraeten y van Der Straeten, 2017; Yoon y Kieber, 2013) o incluso que exista una competencia por el sustrato de la enzima ACS, la SAM. Respecto a esto último, se sabe que las poliaminas utilizan también este sustrato y tienen un producto común con el etileno, el MTA. Las poliaminas son compuestos nitrogenados de bajo peso molecular pero de alta actividad biológica, ya que están involucradas en un sinfín de procesos fisiológicos. Las principales poliaminas que nos encontramos en plantas superiores son putrescina (Put), espermidina (Spd) y espermina (Spm), las cuales están involucradas en procesos de floración, desarrollo del fruto, senescencia y también respuesta a estrés, entre otros (Chen et al., 2019; Mustafavi et al., 2018). Además de compartir precursor y producto, también se ha demostrado que las poliaminas afectan a la síntesis de etileno a nivel de expresión génica de la enzima ACS (Li et al., 1992; Wang et al., 2019) y viceversa, el etileno también inhibe las enzimas de biosíntesis de las poliaminas (Icekson et al., 1985). Es por esto que se evidencia un *cross-talk* entre las poliaminas y el etileno, sugiriendo que, si la SAM se está utilizando para la síntesis de poliaminas, la producción de etileno va a ser más bien baja y quizás esto es lo que podría estar ocurriendo en "Flor d'Hivern". Por lo tanto, sería interesante realizar nuevos estudios para definir cómo cambian las poliaminas en esta variedad y ver si se cumple esta hipótesis.

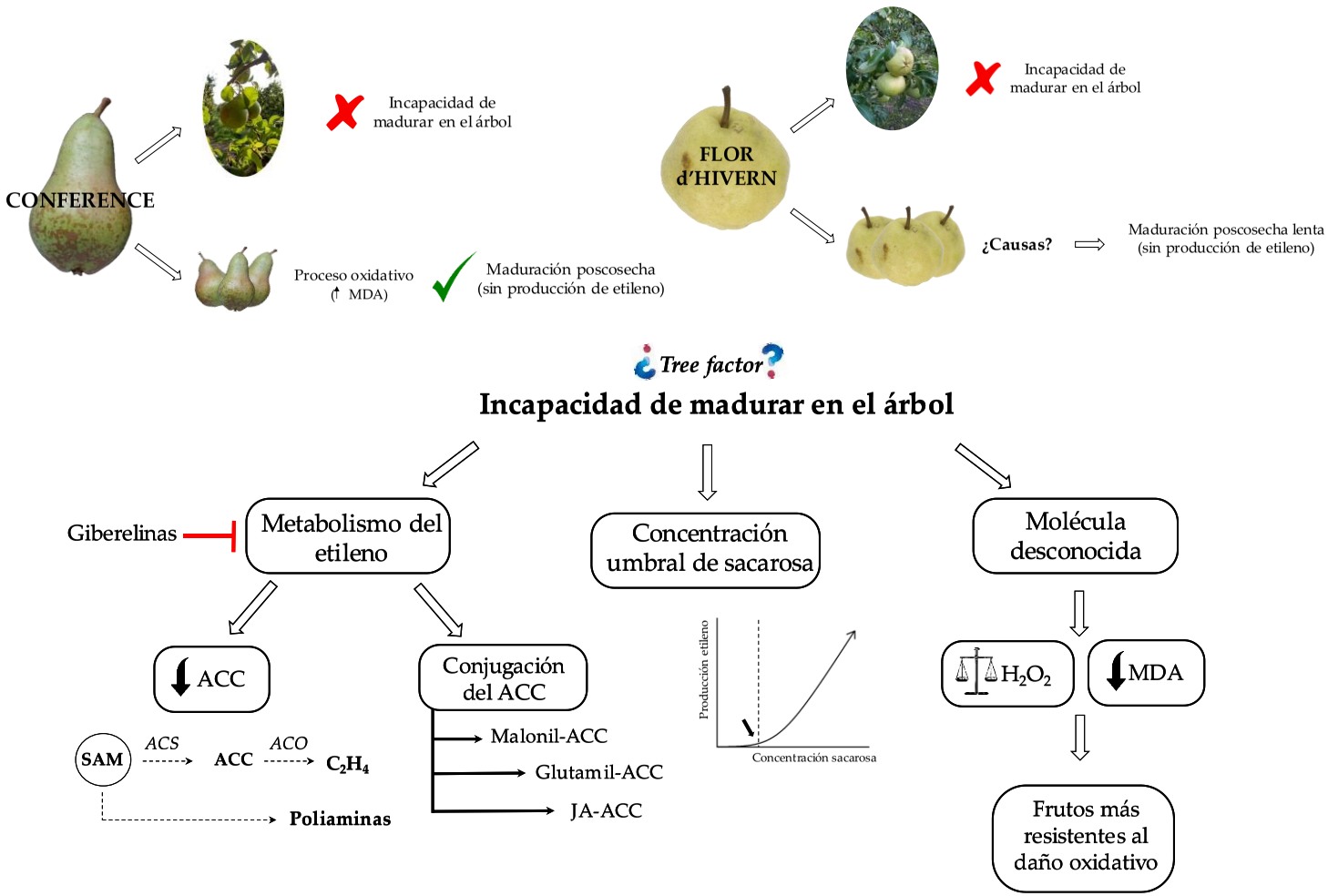


Figura 3. Esquema de los patrones de maduración de "Conference" y "Flor d'Hivern" y los posibles motivos de la incapacidad de madurar en árbol de ambas variedades

El patrón de maduración poscosecha que presentó "Flor d'Hivern" es atípico y teniendo en cuenta los resultados obtenidos nos surge la duda: ¿Y si "Flor d'Hivern" fuera en realidad un fruto no climatérico? Aunque al principio suene un tanto extraño, ya existen algunos estudios en pera asiática donde se habla de variedades climatéricas y no climatéricas, ya que estas últimas ni producen etileno ni responden al tratamiento con propileno (Downs et al., 1991; Yamane et al., 2007) y también otros estudios con melón (Fernández-Trujillo et al., 2008; Périn et al., 2002) y ciruela (Farcuh et al., 2018; Minas et al., 2015) que muestran comportamientos similares. En el estudio de Minas et al. (2015) se hizo una clasificación de las ciruelas en: climatéricas, climatéricas suprimidas y no climatéricas, en función de su patrón de ablandamiento, producción de etileno y de cómo respondían a tratamiento exógeno de propileno. No nos pararemos a comentar las climatéricas, las cuales presentan los picos de etileno y respiración esperados. Sin embargo, sí llama la atención la existencia de algunas variedades que tienen una maduración más lenta y un retraso en la producción de los picos climatéricos de etileno y CO_2, probablemente debido a una capacidad limitada de transformar el ACC en etileno (Abdi et al., 1997), pero que, aunque de manera retardada, sí responden al tratamiento con propileno. Por otro lado, en el mismo estudio de Minas et al. (2015) se mostraron algunas variedades que pueden ser consideradas no climatéricas ya que no producían nada de etileno en cosecha, ni si quiera tras la aplicación de propileno, y con pérdidas de firmeza muy baja o prácticamente inexistente. Aunque no se muestre en esta tesis, en nuestro grupo de laboratorio también se han realizado tratamientos con etileno (200 $\mu L\ L^{-1}$) en "Flor d'Hivern" y no se ha observado que dicho tratamiento tenga efecto sobre la producción de etileno ni sobre las actividades enzimáticas de ACS y ACO. Por lo tanto, basándonos en nuestros resultados junto con los mencionados anteriormente en ciruela, pera japonesa y melón, podríamos pensar que en pera europea también existe alguna variedad con un comportamiento no climatérico y, por qué no, una de estas variedades podría ser "Flor d'Hivern".

3. En busca de un marcador en cosecha capaz de predecir la susceptibilidad al escaldado

Una vez que ya hemos hablado de los diferentes eventos que tienen lugar durante el crecimiento y desarrollo del fruto y la posterior maduración tanto en árbol como después de cosecha, ahora nos centraremos en el desarrollo del escaldado superficial y los diferentes aspectos que pueden afectar a su aparición (Fig. 4). En el Capítulo 4 de la presente tesis se evaluó la incidencia del escaldado superficial en diferentes

estados de madurez de dos variedades muy susceptibles (“Blanquilla” y “Flor d’Hivern”) y su relación con el metabolismo del etileno y componentes antioxidantes.

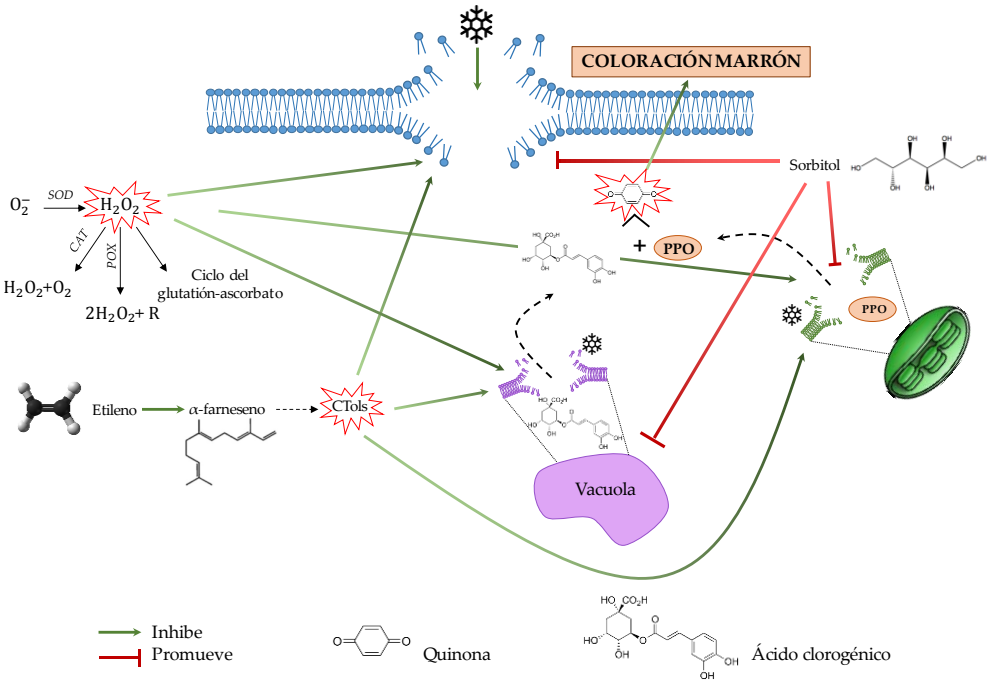


Figura 4. Esquema de los diferentes parámetros que afectan a la aparición del escaldado superficial: I) por un lado las ROS, si no son degradadas, pueden alterar la estabilidad de las membranas, II) el etileno favorece la síntesis de α-farneseno, el cual es oxidado a CTols que también pueden desestabilizar las membranas, III) el frío promueve la rotura de las membranas, haciendo que se pongan en contacto la polifenoloxidasa (PPO) con el ácido clorogénico produciéndose quinonas, las cuales están involucradas en la aparición de la coloración marrón y IV) el efecto crioprotector que ejerce el sorbitol sobre las diferentes membranas celulares. Figura creada con BioRender.com.

La pera “Blanquilla” sigue en general el modelo que se suele utilizar para esta alteración fisiológica en peras. En él, se relaciona la incidencia de la fisiopatía con el estado de madurez del fruto (a mayor madurez, más incidencia) tal y como se ha demostrado para otras variedades de pera europea (Calvo et al., 2015). También se relaciona con la capacidad de producción de etileno (la fruta más madura necesita menos días para alcanzar el pico climatérico) y capacidad de acumulación α-farneseno mediante cambios de la expresión génica (aumento de la expresión del gen *PcAFS1*), tal y como se ha descrito en otros estudios con manzana (Pechous et al., 2005) y pera (Gapper et al., 2006). En el Capítulo 4 se observó un metabolismo del

etileno bastante acorde a este modelo en pera "Blanquilla", ya que la mayor o más temprana producción de etileno estaba relacionada con un mayor contenido en ACC y éste con una mayor actividad ACS. Por otro lado, la actividad ACO se mantuvo constante a lo largo del tiempo, lo cual nos indica que probablemente el paso limitante en la producción de etileno en esta variedad es el paso de SAM a ACC mediante la acción de la ACC sintasa (Yang y Hoffman, 1984).

Por otro lado tenemos la variedad "Flor d'Hivern", la cual mostró un porcentaje de fruta afectada muy alto, incluso nada más salir de la conservación en frío. Este hecho parece ser independiente del etileno, ya que los frutos no produjeron etileno ni siquiera después de un mes de almacenamiento en frío. Esta falta de producción de etileno podría deberse a una baja disponibilidad del sustrato ACC, a pesar de que la actividad ACS es considerable, y la ACO muestra niveles incluso mayores que los observados en "Blanquilla". Ante este escenario y teniendo también en cuenta los resultados obtenidos para α-farneseno y $CTol_{281}$, donde no se observaba una relación clara con el escaldado superficial, se decidió orientar el estudio hacia el metabolismo oxidativo y de ahí que se analizasen algunos de los principales compuestos y enzimas antioxidantes involucrados.

A pesar de que la implicación del α-farneseno y sus productos de oxidación en la aparición del escaldado superficial es una teoría bien estudiada, algunas investigaciones apuntan más hacia la importancia del sistema antioxidante del fruto para explicar este desorden. De hecho, la incidencia del escaldado superficial no se correlaciona siempre con los niveles de α-farneseno (Larrigaudière et al., 2016; Rao et al., 1998; Zhang y Shu, 2003). En manzana, algunos autores han demostrado que, a diferencia de lo que se ha creído durante muchos años, los productos de oxidación del α-farneseno no son esenciales para que el fruto desarrolle escaldado superficial si su capacidad antioxidante está disminuida o dañada (Whitaker et al., 2000). Sin embargo, el principal problema de asociar la menor capacidad antioxidante del fruto con una mayor incidencia o susceptibilidad al escaldado superficial es que dicha capacidad depende de muchos factores como la variedad, las condiciones agro-meteorológicas, la fecha de cosecha y las condiciones de almacenamiento (Calvo et al., 2018). En nuestro caso en "Blanquilla", y a pesar de estar bien correlacionado con la producción de etileno y la actividad del gen de la α-farneseno sintasa, un mayor contenido en ascorbato en la primera fecha de cosecha también podría estar relacionado con una mayor resistencia al escaldado superficial. En este punto hay que destacar la importancia del ciclo del glutatión/ascorbato en el desarrollo del escaldado

superficial, ya que se ha demostrado que una baja expresión de las DHAR (enzimas encargadas de formar ácido ascórbico a partir de dehidroascorbato) podría estar relacionada con el desarrollo del escaldado superficial en pera (Wang et al., 2018). Por otra parte, también se ha demostrado que la fruta tratada con 1-MCP tiene sobre expresados los genes de las enzimas APX, DHAR y MDHAR en comparación con la fruta que muestra escaldado (Giné-Bordonaba et al., 2020). Y, por último, también se ha visto que aquellas variedades de pera con una mayor resistencia a desarrollar escaldado superficial, muestran un mayor contenido en ascorbato (Larrigaudière et al., 2016). Todo esto demuestra que los diferentes compuestos y enzimas del ciclo del glutatión/ascorbato podrían ser clave en el desarrollo de la alteración fisiológica, probablemente debido a la participación que tienen dichos compuestos en mantener el balance redox y prevenir un daño oxidativo (Maruta y Ishikawa, 2018; Zuccarelli y Freschi, 2018).

Siguiendo con otras enzimas antioxidantes, una mayor actividad POX también se ha relacionado con una menor susceptibilidad al escaldado en diferentes selecciones de manzana (Fernández-Trujillo et al., 2003; Kochhar et al., 2003; Rao et al., 1998), hecho que también está correlacionado en nuestro estudio al observarse una mayor actividad POX en la fecha de cosecha con menor incidencia de escaldado. Por lo tanto, podríamos decir que en el caso de nuestra pera "Blanquilla" un descenso en el contenido de ascorbato y de la actividad POX podría estar relacionado, junto con el efecto del α-farneseno, con una mayor susceptibilidad al escaldado superficial.

En el caso de la variedad "Flor d'Hivern", el estudio de la capacidad antioxidante y de los diferentes compuestos y enzimas antioxidantes resulta de gran interés ya que, en este caso, no se encontró relación entre la aparición del escaldado y la producción de etileno ni con el contenido de α-farneseno. Sin embargo, los resultados obtenidos fueron incluso más complejos que los de "Blanquilla", ya que, por ejemplo, se observó una mayor incidencia de escaldado a pesar de tener más capacidad antioxidante y un mayor contenido en ascorbato. Esto quizás pueda deberse a que existan otros factores que tengan más peso en el desarrollo de esta alteración en "Flor d'Hivern", como por ejemplo la lipoxigenasa (LOX). La LOX está muy relacionada con la peroxidación lipídica de las membranas, ya que sus sustratos son principalmente ácidos grasos poliinsaturados. De hecho, algunos estudios realizados con pera japonesa han relacionado una mayor actividad de LOX con procesos de senescencia y desarrollo de escaldado (Li y Wang, 2009; Liu et al., 2013; Zhou et al., 2016).

Respecto a la capacidad antioxidante del fruto es necesario remarcar un par de cosas: I) se considera que es más importante la acción individualizada de una enzima que el balance de todas las enzimas antioxidantes o la capacidad antioxidante global, tal y como se ha establecido tanto en manzana (Kochhar et al., 2003) como en pera (Larrigaudière et al., 2016), y II) es más importante la acumulación de los compuestos antioxidantes durante el almacenamiento en frío que sus valores en cosecha (Calvo et al., 2015). Teniendo en cuenta esto, y lo que comentamos anteriormente sobre la multitud de factores que pueden determinar la capacidad antioxidante del fruto, es fácil entender la dificultad para establecer un marcador del escaldado superficial en cosecha que sea capaz de predecir este desorden en diferentes variedades o regiones. Sin embargo, nuestros resultados en pera "Blanquilla" indicaron que la enzima ACS y el metabolito ACC estaban positivamente correlacionados con la incidencia de la fisiopatía (r^2 = 0,63 y 0,71 con $p \leq 0,05$, respectivamente), por lo que podrían ser marcadores interesantes del escaldado en esta variedad. Además, también se podría considerar como marcador el gen *PcAFS1*, ya que también muestra una correlación positiva (r^2 = 0,59 con $p \leq 0,05$) con la aparición del desorden. Por otro lado está la variedad "Flor d'Hivern", en la que no se encontraron buenas correlaciones con los parámetros estudiados; lo cual nos indica que harían falta más estudios metabólicos y moleculares con el fin de poder encontrar un marcador específico para esta variedad.

Dentro de este capítulo también se remarcó la importancia del sorbitol; este es un polialcohol y, junto con la sacarosa y el almidón, uno de los principales productos de la fotosíntesis en la mayoría de los miembros de las rosáceas (Wallaart, 1980). Algunos estudios apuntan hacia la capacidad crioprotectora de este compuesto (Lloret et al., 2017; Moing et al., 1997) y se han obtenido resultados interesantes en plantas de Arabidopsis (Busatto et al., 2018) y pera "Blanquilla" (Giné-Bordonaba et al., 2020). Es por esto que en el Capítulo 4 se lanzó la hipótesis de que los menores niveles de sorbitol observados en "Flor d'Hivern", en comparación con "Blanquilla", podrían estar relacionados con una menor tolerancia a los daños por frío y de ahí la aparición de esta fisiopatía incluso en el frío.

Teniendo en cuenta los resultados y las correlaciones existentes entre algunos de los parámetros estudiados, podemos decir que el modelo del escaldado superficial en pera "Blanquilla" sigue el modelo estándar que relaciona la alteración con el etileno y el α-farneseno. Al realizar un análisis de regresión de mínimos cuadrados se puede decir también, en esta variedad, el ascorbato fue la variable que mejor predijo la

aparición del escaldado superficial con una relación negativa y un valor del estadístico VIP (de sus siglas en ingés, *Variable Influence on Projection*) superior a 1,1. Considerando que este compuesto actúa como antioxidante, queda destacada la importancia del ascorbato en la alteración. Sin embargo, en "Flor d'Hivern" la relación con el metabolismo del etileno y el α-farneseno no queda tan clara, y además resulta difícil encontrar algún parámetro que esté íntimamente relacionado con la aparición de la alteración. La apariencia del desorden se asemeja bastante al escaldado de senescencia, pero la pulpa no se ve afectada ni en textura ni en color; por lo tanto, quizás nos deberíamos plantear que la fisopatía que muestra "Flor d'Hivern" es un daño por frío clásico más que un típico escaldado superficial, aunque harían falta más estudios con el fin de poder confirmar esta hipótesis.

4. Influencia del metabolismo del etileno y el α-farneseno en el desarrollo del escaldado superficial

Una vez estudiados ciertos parámetros en cosecha, en el Capítulo 5 se decidió estudiar el metabolismo del etileno y del α-farneseno y sus interacciones durante los primeros meses de almacenamiento en frío, periodo que se corresponde con la fase de inducción del escaldado superficial (Lurie y Watkins, 2012). Para saber de qué manera estaban involucradas estas vías metabólicas, se trató los frutos con dos inhibidores: uno del etileno (el 1-MCP) y otro del α-farneseno (la lovastatina) conocidos por reducir la incidencia de escaldado en manzanas y peras (Giné-Bordonaba et al., 2020; Ju and Curry, 2000a; Larrigaudière et al., 2019; Zhi and Dong, 2018). Tal y como se mencionó anteriormente, la mayoría de estudios están orientados en definir los cambios producidos durante la vida útil después de una conservación más o menos larga (Giné-Bordonaba et al., 2020; Zhou et al., 2020), pero pocos estudios hay sobre los cambios que se producen durante las primeras semanas de almacenamiento.

El 1-MCP, como ya se mencionó en la introducción, se une a los receptores del etileno de manera irreversible e inhibe la biosíntesis del mismo. Es de los compuestos más utilizados y estudiados para mejorar la calidad poscosecha de una gran variedad de cultivos, de ahí el gran número de artículos donde lo mencionan (Blankenship y Dole, 2003; Watkins, 2008, 2006; Larrigaudière et al. 2019; Giné-Bordonaba et al. 2020). Algunos estudios demuestran que este producto inhibe la producción de etileno en peras así como también su metabolismo (a nivel de ACS, ACC y ACO; Chiriboga et al., 2012), y la expresión génica asociada (genes de enzimas de biosíntesis y de la ruta

de señalización; Chiriboga et al., 2013). Al fijarse de forma competitiva a nivel de los receptores del etileno, el 1-MCP impide la acción de este último sobre los procesos de señalización e inducción de la maduración (Gamrasni et al., 2010; Liu et al., 2015; Villalobos-Acuña et al., 2011; Fig. 5). El efecto inhibidor del 1-MCP sobre el etileno, también hace que se vean afectados todos los procesos regulados por la hormona; de ahí que también se observe una inhibición de la acumulación de α-farneseno y CTols (Gapper et al., 2006; Isidoro y Almeida, 2006; Li y Wang, 2009) y de la expresión de los genes de su ruta de síntesis: *PcAFS1* y *PcHMGR* (Zhou et al., 2017). A nivel de percepción y señalización, el 1-MCP inhibe la expresión del gen *PcERF1* (de sus siglas en inglés: *Ethylene Response Factor 1*), el cual codifica para un factor de transcripción encargado de desencadenar toda la repuesta del etileno cuando éste se une a sus receptores (Chang, 2016). El 1-MCP también promueve la expresión de los genes *PcETR1* (*Ethylene Receptor 1*) y *PcEIN2* (*Ethylene insensitive 2*). Esta inducción podría estar relacionada con la regulación negativa de los receptores, ya que estos son degradados como respuesta a la unión de etileno (Chen et al., 2007), suponiendo así que, en ausencia de etileno, se esté promoviendo la síntesis de más receptores.

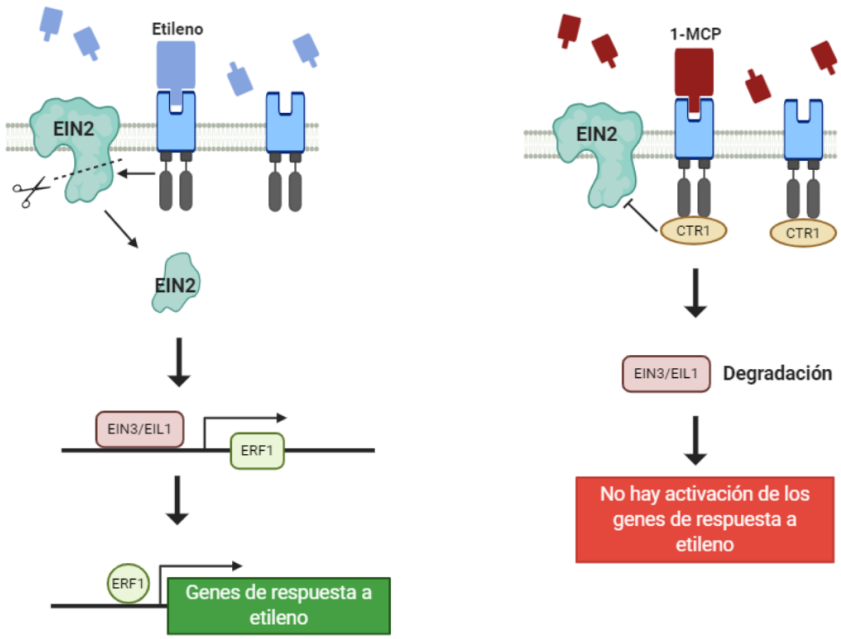


Figura 5. Modo de acción del 1-MCP. Si el etileno se une a sus receptores (ETR1) se favorece la degradación proteolítica de la proteína EIN2, la cual promueve factores de transcripción que activan los genes de respuesta a etileno (A). Por el contrario, si no hay etileno y es el 1-MCP quien se une a los receptores, se activa el componente CTR1 (*Constitutive Response 1*), una

proteína con actividad quinasa que inhibe la proteólisis de EIN2, provocando la degradación de los factores de transcripción que esta proteína activaba e inhibiéndose así todos los procesos posteriores que se desencadenaban (B). Figura creada con BioRender.com.

Respecto al desarrollo de escaldado superficial, el 1-MCP inhibió casi en su totalidad la incidencia de escaldado superficial en "Blanquilla" y "Flor d'Hivern" pero no en la variedad "Conference". El efecto positivo de este tratamiento en "Blanquilla" ya ha sido mostrado en estudios previos (Gamrasni et al., 2010; Giné-Bordonaba et al., 2020; Larrigaudière et al., 2019) y en el caso de "Flor d'Hivern", se suma a la gran lista de variedades sobre las que tiene efecto el 1-MCP (Almeida et al., 2016; Argenta et al., 2003; Gao et al., 2015; Villalobos Acuña et al., 2011; Xie et al., 2014). En el caso de "Conference", a pesar de inhibir la producción de etileno, no inhibió el desorden sino que incluso lo promovió. *A priori* este resultado sorprende, pero nos hace pensar que la alteración que se observa en "Conference" no es un escaldado superficial típico sino más bien otro tipo de desorden con una etiología diferente. De hecho, en un estudio llevado a cabo por Rizzolo et al. (2015) en pera "Conference" no detectaron peras afectadas por escaldado superficial tras 4 meses de almacenamiento en frío, pero sí por otros tipos de alteraciones que llamaron *blackening* y "peca negra" (*black speck)*. Además, comprobaron que el tratamiento con 1-MCP no era capaz de controlar estas alteraciones e incluso que, en algunos casos, aumentaba su incidencia. Resultados similares también se han obtenido con pera asiática, donde el tratamiento con 1-MCP aumentó la incidencia de mancha negra (*black spot*), aunque en este caso no se observó inhibición de la producción de etileno (Itai et al., 2012).

El otro tratamiento utilizado, la lovastatina, afecta a la síntesis de α-farneseno mediante la inhibición de la enzima HMGR pero sin afectar a la producción de etileno (Giné-Bordonaba et al., 2020; Ju y Curry, 2000b, 2000a). En "Blanquilla", la lovastatina redujo considerablemente los niveles de α-farneseno y $CTol_{281}$ y también controló de manera muy eficaz la incidencia de escaldado superficial sin alterar el metabolismo del etileno. Este hecho refuerza la hipótesis de que la aparición del escaldado superficial en esta variedad está altamente ligado al metabolismo del α-farneseno y que el etileno por sí solo no determina la incidencia del desorden. Al igual que ocurría con el 1-MCP, la lovastatina tampoco controló la aparición de la alteración en "Conference", a pesar de reducir considerablemente los niveles de α-farneseno, lo que refuerza nuestra hipótesis sobre la presencia de una alteración con etiología diferente al escaldado superficial.

Lo que más llamó la atención de este estudio es el comportamiento atípico que presenta la variedad "Flor d'Hivern". A lo largo de los últimos capítulos se ha visto que esta variedad no sigue las reglas "normales" establecidas para la maduración e incluso para la aparición del escaldado superficial, ya que esta variedad pierde firmeza y cambia de color una vez ha sido cosechada sin producir etileno ni observarse, *a priori*, daños en la membrana (Capítulo 3). Las peras "Flor d'Hivern" muestran la fisiopatía nada más salir de la conservación en frío, sin poder observar diferencias de incidencia entre las diferentes fechas de cosecha y sin producir etileno (Capítulo 4). Además, a pesar de ser una variedad muy susceptible al escaldado superficial presenta una elevada capacidad antioxidante, un alto contenido en ascórbico (Capítulo 4) y ahora como se ha visto en el Capítulo 5, no produce etileno ni después de 60 días de almacenamiento. El tratamiento con 1-MCP es capaz de inhibir la aparición del escaldado en esta variedad pero no la lovastatina, la cual tampoco reduce los niveles de α-farneseno. Por lo tanto, esto indica que la etiología de la alteración en esta variedad es distinta a la de "Blanquilla" ya que, en "Flor d'Hivern", el escaldado no está ligado al etileno ni al α-farneseno, sino más bien a un proceso oxidativo. Sin embargo, si el desorden fisiológico que vemos en "Flor d'Hivern" no está relacionado con el etileno, ¿Cómo es posible que el tratamiento con 1-MCP inhiba de manera muy eficaz la aparición de escaldado en esta variedad? De primeras puede extrañar ya que, como ya sabemos, el 1-MCP es un inhibidor de la respuesta del etileno; pero algunos estudios han demostrado que también afecta a otras rutas en las que está involucrado diferentes enzimas y compuestos del metabolismo antioxidante. Du et al. (2017) demostraron que en manzanas tratadas con 1-MCP se veía una mayor actividad enzimática de la sorbitol-6-fosfato deshidrogenasa (S6PDH, encargada de la síntesis de sorbitol) y de la POX y una disminución de la actividad enzimática de LOX. La implicación del metabolismo del sorbitol en el desarrollo del escaldado superficial también fue demostrada recientemente en pera "Blanquilla", donde se observó que la fruta tratada con 1-MCP tenía mayor contenido en sorbitol y una mayor expresión génica de la S6PDH (Giné-Bordonaba et al., 2020). Además, otros estudios en pera han demostrado que este tratamiento también es capaz de mantener los niveles de glutatión y los ratio redox (Flaherty et al., 2018) y aumentar la expresión génica de enzimas involucradas en el ciclo del glutatión-ascorbato (Giné-Bordonaba et al., 2020; Wang et al., 2018). Considerando todo esto y teniendo en cuenta los resultados del Capítulo 4, podríamos pensar que el 1-MCP inhibe la aparición del escaldado superficial en esta variedad actuando sobre las enzimas del metabolismo antioxidante, probablemente disminuyendo la actividad LOX, potenciando la POX y activando la síntesis de

sorbitol; en un conjunto que finalmente incrementa el potencial de aclimatación al frío del fruto. Algunos estudios recientes en pera y manzana han demostrado que la capacidad de un fruto para prevenir el escaldado superficial está relacionada con el proceso de aclimatación, el cual le confiere cierta tolerancia al frío (Busatto et al., 2018; Giné-Bordonaba et al., 2020). Una de las respuestas de este proceso es promover la síntesis de compuestos que estabilizan las membranas (como sorbitol, ácidos grasos de cadena larga, etc.) e impedir los procesos de permeabilización de las membranas que a largo plazo provocan los problemas de pardeamiento y el escaldado superficial.

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CONCLUSIONES

El objetivo principal de esta tesis era el estudio de las bases fisiológicas, bioquímicas y moleculares de la maduración en peras y su relación con el desarrollo del escaldado superficial. Para lograrlo, se establecieron unos objetivos específicos que se han ido discutiendo a lo largo de los diferentes capítulos y de los cuales se han podido extraer las siguientes conclusiones:

<u>Objetivo 1</u>. Definir cómo las hormonas endógenas y sus interacciones (*cross-talk*) durante el desarrollo del fruto en campo definen las características cualitativas y el potencial de maduración de diferentes cultivares de pera.

Conclusiones:

1.1 A pesar de que siempre se le reconoce como la hormona principal, el etileno no es la única hormona que está implicada en el desarrollo y la maduración de la pera.

1.2 La regulación hormonal existente durante el desarrollo y posterior maduración del fruto depende de la variedad de pera estudiada.

1.3 Más que la acción individualizada de una hormona, podemos decir que es el *cross-talk* hormonal existente el responsable del correcto desarrollo y maduración del fruto; y que el balance de estas hormonas determinan el posterior comportamiento poscosecha.

1.4 Las hormonas ácido abscísico (ABA), ácido 3-indolacético (IAA) y giberelina 1 (GA_1) juegan un papel determinante no sólo en el desarrollo del fruto sino también en su potencial de maduración. El ABA y el IAA actúan como inductores de la maduración en peras fomentando la síntesis de etileno a través de su acción inductora sobre las enzimas ACS y ACO. Por otro lado, las giberelinas regulan de manera negativa los genes de biosíntesis del etileno, por lo que podrían retrasar o inhibir la maduración y explicar la incapacidad de ciertas peras (peras de invierno) de madurar sin recibir un periodo a bajas temperaturas.

1.5 De las interacciones más remarcables está la del ABA con la sacarosa, y a su vez con el etileno, siendo todos estos compuestos de vital importancia en el desarrollo y maduración del fruto.

<u>Objetivo 2</u>. Estudiar desde un punto de vista fisiológico, bioquímico y cualitativo, el patrón de maduración en el árbol y fuera de él de distintas variedades de pera (peras de verano e invierno).

Conclusiones:

2.1 A diferencia de otras peras europeas, "Blanquilla" es una variedad capaz de madurar en el árbol, alcanzando niveles de madurez aptos para el consumo mientras que "Conference" y "Flor d'Hivern" no.

2.2 La capacidad de maduración en el árbol de "Blanquilla" parece estar relacionada con una acumulación de sacarosa, la cual es a su vez capaz de inducir la producción de etileno posiblemente a través de la interacción sacarosa-ABA mencionada anteriormente.

2.3 La maduración poscosecha (*off-tree*) de "Blanquilla" y "Conference" se inicia con un proceso oxidativo en los días inmediatamente posteriores a la cosecha. Este proceso oxidativo podría desencadenar la posterior maduración etileno-dependiente, observada típicamente en la variedad "Blanquilla".

2.4 La incapacidad de madurar en árbol de las variedades "Conference" y "Flor d'Hivern" se relacionan con: I) niveles bajos de sacarosa que son insuficientes para activar la producción de etileno, II) niveles bajos de ACC que podrían estar relacionados con un incremento de la formación de formas conjugadas o derivación hacia otra ruta metabólica, III) mantenimiento de un alto potencial de defensa contra el estrés oxidativo (altos niveles de H_2O_2 y bajos de MDA) y IV) altos niveles de giberelinas que inhiben la producción de etileno.

2.5 El extraño comportamiento observado en "Flor d'Hivern" a lo largo de los diferentes ensayos realizados en esta tesis, indican que tal vez se trate de una variedad de tipo no climatérico, tal y como se ha descrito en otras especies de pera asiática, así como también en melón y ciruela.

<u>Objetivo 3</u>. Identificar un marcador bioquímico o molecular en cosecha capaz de predecir la susceptibilidad al escaldado superficial.

Conclusiones:

3.1 El escaldado superficial en "Blanquilla" se puede atribuir en gran medida al modelo estándar que relaciona la incidencia con la relación α-farneseno-etileno, mientras que en "Flor d'Hivern" estos compuestos parecen no tener un papel principal.

3.2 Los niveles de expresión del gen *PcAFS1*, junto con el contenido en ACC y la actividad ACS parecen ser buenos marcadores en cosecha para predecir la susceptibilidad de la pera "Blanquilla", observándose una relación positiva entre estos compuestos y el escaldado superficial. La enzima POX y los niveles de ascórbico parecen ser también unos marcadores segundarios interesantes para la predicción en cosecha.

3.3 Los mejores predictores en pera "Flor d'Hivern" no se relacionan con el metabolismo del etileno, pero parece que una mayor actividad LOX y unos niveles de sorbitol bajos podrían estar relacionados con el desorden en esta variedad.

<u>Objetivo 4</u>. Redefinir el rol específico que juegan el etileno y el α-farneseno en el desarrollo del escaldado superficial para ver cómo estas vías metabólicas se integran en el modelo explicativo del desorden en pera.

Conclusiones:

4.1 La variedad "Blanquilla" muestra un escaldado superficial claramente relacionado con la capacidad del fruto de producir etileno y, por lo tanto, de regular la expresión del gen *PcAFS1*.

4.2 A pesar de que "Flor d'Hivern" es una variedad incapaz de producir etileno ni en cosecha ni tras un periodo de almacenamiento en frío, el tratamiento con 1-MCP en "Flor d'Hivern" parece mejorar la capacidad de aclimatación al frío del fruto, controlando así de manera eficaz la aparición del escaldado.

4.3 Considerando los diferentes resultados obtenidos en "Flor d'Hivern" en el Capítulo 4, junto con el hecho de que la lovastatina no reduce los niveles de α-farneseno ni inhibe el escaldado en esta variedad, es probable que el desorden fisiológico observado sea un desorden fisiológico provocado por un daño por frío en el cual se ve afectado sobre todo la integridad de las membranas.

4.4 La ineficacia del 1-MCP y lovastatina en "Conference" podría indicar la presencia de una alteración con etiología diferente al escaldado superficial (desorden tipo *blackening*).

4.5 A nivel molecular, la expresión de los genes *PcACS1* y *PcACO1* se ven claramente suprimidas por el 1-MCP, pero no por la lovastatina. En los genes de la ruta de percepción y señalización, el 1-MCP induce la expresión de *PcEIN2* y *PcETR1* y reprime la de *PERF1*, mientras que la lovastatina provoca el efecto contrario.

4.6 La lovastatina no afecta a la expresión génica de *PcHMGR* en "Blanquilla" pero reduce su expresión en las variedades "Conference" y "Flor d'Hivern"; por otro lado, este tratamiento induce la expresión de *PcAFS1* en las tres variedades, siendo más notable en la variedad "Blanquilla". El 1-MCP reprime la expresión de ambos genes en las tres variedades estudiadas.

PERSPECTIVAS FUTURAS

Los resultados y conclusiones de la presente tesis abren nuevos interrogantes que deberán ser resueltos en futuros trabajos. Empezando por el *cross-talk* observado durante el desarrollo y la maduración del fruto, sería interesante continuar el estudio del perfil hormonal durante la maduración en árbol y fuera de árbol, con el fin de determinar cómo evolucionan las hormonas una vez la fruta ha alcanzado su madurez fisiológica. También se podría trabajar con tratamientos hormonales para ver si aplicaciones de ABA o IAA en campo o tras la cosecha son capaces de promover la producción de etileno en una variedad como por ejemplo "Conference". También resultaría de gran interés evaluar el perfil hormonal, tanto durante el desarrollo en campo como durante la maduración poscosecha de la variedad "Flor d'Hivern" para ver si los niveles de hormonas explican la incapacidad de producir etileno que caracteriza esta variedad.

Respecto al patrón de maduración en el árbol, sería interesante seguir con el estudio del *tree factor* para llegar a conocer qué molécula o moléculas son las responsables de inhibir la maduración del fruto. Para ello, haría falta realizar el mismo tipo de estudio en otras variedades y determinar hasta qué punto la sacarosa está involucrada en la capacidad de producción de etileno, qué antioxidantes están involucrados en la resistencia a madurar en el árbol y finalmente definir qué procesos oxidativos se relacionan con la inducción de maduración a corto plazo después de cosecha. Por otro lado, se podría realizar también un análisis metabólico o transcriptómico global comparando variedades capaces de madurar o no en el árbol para así acabar de definir las características del *tree factor*.

Por lo que se refiere al desarrollo del escalado superficial resultaría muy interesante profundizar los estudios que se refieren a las variedades 'Conference' y 'Flor d'Hivern. En el caso de "Conference", queda pendiente definir las bases fisiológicas exactas del desorden que como hemos visto no se relaciona con los niveles de etileno y α-farneseno y no se inhibe con los inhibidores utilizados. Sería también interesante definir si esta alteración se ve afectada por condiciones climáticas o del suelo (esto explicaría por qué en nuestro ensayo, repetido durante 3 campañas consecutivas, no detectamos escaldado superficial mientras que en otras campañas o fruta proveniente de otras localizaciones sí) y precisar qué factores condicionan la aparición de escaldado superficial y este tipo de desorden (mancha negra) en 'Conference'.

Por otro lado tenemos la variedad "Flor d'Hivern", cuya alteración parece estar más relacionada con un proceso oxidativo provocado por un daño por frío más que con

un escaldado superficial típico. En esta variedad sería necesario analizar cómo afectan los dos tratamientos estudiados en esta tesis, sobre todo el 1-MCP, al metabolismo antioxidante del fruto (tanto a nivel bioquímico como molecular). Además, otro tema de estudio bastante importante sería averiguar por qué esta variedad es incapaz de producir etileno, quizás así se podría demostrar la existencia de cultivares no climatéricos dentro de la especie *Pyrus communis*.